British Journal of Pharmacology (2009), 156, 774–785

0007-1188/09
www.brjpharmacol.org

RESEARCH PAPER

Low-dose oral sirolimus reduces atherogenesis, vascular inflammation and modulates plaque composition in mice lacking the LDL receptor

L Zhao[2], T Ding[1], T Cyrus[3], Y Cheng[1], H Tian[4], M Ma[4], R Falotico[2] and D Praticò[1]

[1]*Department of Pharmacology, Temple University School of Medicine, Philadelphia, PA, USA,* [2]*Cordis Corporation, a Johnson and Johnson Company, Warren, NJ, USA,* [3]*Department of Cardiology, Washington University, St. Louis, MO, USA, and* [4]*Department of Neuroscience, University of Pennsylvania, Philadelphia, PA, USA*

Background and purpose: Chronic proliferative responses of different vascular cell types have been involved in the pathogenesis of atherosclerosis. However, their functional role remains to be established. Sirolimus reduces neointimal proliferation after balloon angioplasty and chronic graft vessel disease. These studies were undertaken to investigate the effects of this anti-proliferative drug on atherogenesis.
Experimental approach: Low-density lipoprotein receptor-deficient (LDL r-KO) mice on a cholesterol-rich diet were randomized to receive placebo or sirolimus (0.1; 0.3; or 1 mg·kg^{-1}) in their diet for 8 or 16 weeks.
Results: In both studies, plasma levels of the drug increased in a dose-dependent fashion, animals gained weight normally and, among groups, plasma lipids levels did not differ significantly. Compared with placebo, plasma levels of interleukin-6, monocyte chemoattractant protein-1, interferon γ, tumour necrosis factor α and CD40, and their mRNA levels in aortic tissue were significantly reduced in sirolimus-treated mice. This effect resulted in a significant and dose-dependent reduction in atherosclerotic lesions, in both the root and aortic tree. Also these lesions contained less monocyte/macrophages and smooth muscle cells, but more collagen.
Conclusions and implications: The present results demonstrated that at low doses, sirolimus was an effective and safe anti-atherogenic agent in the LDL r-KO mice. It attenuated the progression of atherosclerosis and modulated the plaque phenotype by reducing the pro-inflammatory vascular responses typical of the disease.
British Journal of Pharmacology (2009) **156**, 774–785; doi:10.1111/j.1476-5381.2008.00080.x; published online 13 February 2009

Keywords: atherosclerosis; animal models; vascular inflammation

Abbreviations: 2,3dinor 6-oxo-$PGF_{1\alpha}$, 2,3 dinor 6keto-prostaglandin $F_{1\alpha}$; 2,3dinor TxB_2, 2,3 dinor thromboxane B_2; IFN-γ, interferon γ; IL-6, interleukin-6; LDL r-KO, low-density lipoprotein receptor-deficient; MCP-1, monocyte chemoattractant protein-1; TNF-α, tumour necrosis factor α

Introduction

Atherosclerosis is a chronic disease of the vasculature influenced by multiple factors and involves a complex interplay between components of the blood and the arterial wall. Evidence consistently supports the hypothesis that immunological and inflammatory responses play an important role at every stage during the development of atherosclerosis (Greaves and Channon, 2002). The disease process develops and progresses in response to abnormal cholesterol deposits in the intima of large arteries. It is initiated by transendothelial migration and activation of circulating monocytes and lymphocyes at the site of vessel injury. The recruited cells then release potent inflammatory chemokines and cytokines, which promote vascular smooth muscle cell proliferation and migration towards the atherosclerotic lesion, further increasing the neointimal thickening. During these processes both adaptive and innate immune mechanisms can modulate the inflammatory vascular phenotype typical of the disease (Libby, 2002; Hansson *et al.*, 2003).

Sirolimus is a macrolide antibiotic and a natural fermentation product of *Streptomyces hygroscopicus* with potent immunosuppressive, anti-proliferative and anti-migratory activities (Sehgal *et al.*, 1994; Marks, 2003). As an immunosuppressive agent, sirolimus has been an effective drug for the prevention of renal and cardiac allograft rejection (Snell *et al.*, 2003). Furthermore, in animal studies, sirolimus has been found to significantly reduce neointimal proliferation in response to

Correspondence: Domenico Praticò, Temple University, 3420 North Broad Street, MRB, 706A, Philadelphia, PA 19140, USA. E-mail: praticod@temple.edu
Received 5 August 2008; revised 7 October 2008; accepted 21 October 2008

mechanical injury (Gall *et al.*, 1999; Farb *et al.*, 2002). These findings have resulted in the development of a sirolimus-eluting stent, which has shown positive effects in reducing restenosis and repeat revascularization rates in human coronary arteries compared with standard bare metal stents (Morris *et al.*, 1995; Sousa *et al.*, 2001).

The consistent evidence supporting a role for chronic proliferative reactions in the development of atherosclerosis has generated new interest in sirolimus, as it could also be beneficial for prevention and treatment of this chronic vascular disease. Thus, some studies showed that intra-peritoneal injection of high-dose sirolimus reduced cholesterol content in atherosclerotic lesions in mice, but it also induced an increase in plasma triglyceride and cholesterol levels (Elloso *et al.*, 2003; Castro *et al.*, 2004; Naoum *et al.*, 2005). These unanticipated findings clearly justified a re-evaluation of this drug in order to establish its potential clinical relevance in the setting of atherosclerosis.

To this end, the present studies were designed to test whether by using a much lower dosage of sirolimus administered via the oral route and for a longer time, we could demonstrate a novel anti-atherogenic effect without any influence on the circulating lipids.

Here, we show that, in the low-density lipoprotein receptor-deficient (LDL r-KO) mice, sirolimus was well tolerated, and without influencing total plasma lipid levels, dose-dependently reduced atherogenesis, by selectively modulating inflammatory vascular responses, which ultimately resulted in lesions containing less cells and more collagen.

Methods

Animals and protocols

All procedures and care of animals were approved by the Institutional Animal Care and Usage Committee. LDL r-KO mice (backcrossed 10 times to C57BL/6 background) were purchased from The Jackson Laboratory (Bar Harbor, ME, USA) at 6 weeks of age and housed under pathogen-free conditions. Only male mice were used in the studies presented in this paper. Starting at 8 weeks of age, mice were fed a high-fat diet (normal chow supplemented with 0.2% cholesterol and 21% fat), and randomized to four groups receiving: placebo; sirolimus 0.1 $mg \cdot kg^{-1}$; sirolimus 0.3 $mg \cdot kg^{-1}$; and sirolimus 1 $mg \cdot kg^{-1}$ for 8 or 16 weeks. Each group was comprised of 15 mice. Sirolimus was added to the high-fat diet at the concentrations described above by a commercial vendor (Harlan Teklad, Madison, WI, USA). Mice had free access to food and water during the studies.

Urines were collected overnight in a metabolic cage; blood samples were obtained from animals fasted overnight by retro-orbital bleeding, as previously described (Cyrus *et al.*, 2002).

Biochemical analysis

Urinary 2,3 dinor thromboxane B_2 (2,3dinor TxB_2) and 2,3 dinor 6keto-prostaglandin $F_{1\alpha}$ (2,3dinor 6-oxo-$PGF_{1\alpha}$), isoprostane $iPF_{2\alpha}$-III and creatinine levels were all measured as previously described (Cyrus *et al.*, 2007). Plasma cholesterol and triglyceride levels were determined enzymically using Sigma reagents (Sigma Chem. Co., St Louis, MO, USA) (Cyrus *et al.*, 2007). Plasma levels of sirolimus were assayed by an online solid phase extraction combined with liquid chromatography-tandem mass spectrometry. Briefly, protein was precipitated from the samples with 5% methanol/zinc sulphate solution containing the internal standard ascomycin. After centrifugation, the liquid mixture was extracted with 1-chlorobutane, the organic extract dried down then reconstituted with 50:50 methanol:20 $mmol \cdot L^{-1}$ ammonium acetate solution. The samples were finally analysed on API-5000 using C18 reverse-phase chromatography in Multiple Reaction Monitoring (MRM) mode using the atmospheric pressure chemical ionization approach.

Plasma cytokine determination

Mouse cytokine antibody array I (Raybiotech Inc., Norcross, GA, USA) was used according to the manufacturer's instructions. Briefly, the arrays were blocked, incubated with 100 μL plasma overnight at 4°C, incubated with biotin-conjugated antibodies for 2 h, and with horseradish peroxidase antibodies for 2 h. The membranes were then incubated with chemiluminescent substrate and exposed to X-ray film before development. Quantitative array analysis was performed by using the Image-Pro Plus software.

Preparation of mouse aortas and quantitation of atherosclerosis

After the final blood collection, mice were killed and the aortic tree was perfused for 10 min with ice-cold phosphate-buffered saline (PBS) by inserting a cannula into the left ventricle and allowing free efflux from an incision in the vena cava.

Following removal of the surrounding adventitial tissue, the aorta was opened longitudinally from the aortic root to the iliac bifurcation, fixed in formalin-sucrose then stained with Sudan IV. The extent of atherosclerosis was determined using the 'en face' method. Quantitation was performed by capturing images of aortas with a Dage-MTI 3CCD three chips colour video camera connected to a Leica MZ12 dissection microscope, as previously described (Cyrus *et al.*, 2002; 2007).

Atherosclerosis was also quantified in the aortic root cross-sections from fresh-frozen optimal cutting temperature medium (OCT)-embedded hearts, as previously described (Cyrus *et al.*, 2006; 2007). Briefly, alternate 10 μm frozen sections of the aortic root covering 300 μm of the proximal aorta, starting at the sinus, were fixed, re-hydrated and stained for atherosclerotic lesions. Images were captured digitally with a video camera connected to a Leica microscope and analysed by computerized image analysis (Image Pro Plus, Media Cybernetics). The acquisition of the images and analysis for both methods were always performed without knowledge of the treatments.

Histology and Immunohistochemistry

Briefly, serial 6 μm frozen sections of the aortic root covering 300 μm of the proximal aorta, starting at the sinus, were fixed in 4% paraformaldehyde, re-hydrated and stained for collagen type I and III, as previously described (Cyrus *et al.*, 2006;

2007). Sections were incubated for 90 min in 0.1% Sirius red F3BA (Polysciences Inc.) in saturated picric acid. After rinsing in 0.01 N HCl and distilled water, sections were dehydrated with 70% ethanol and analysed by polarization microscopy. For smooth muscle cells, monoclonal anti-smooth muscle α-actin (clone 1A4, dilution 1:40, Sigma Chemical Co.) was used as the primary antibody followed by a secondary peroxidase-conjugated rabbit anti-mouse antibody (P-0260, dilution 1:300, Dako). Antibody reactivity was detected with use of a Nova red substrate kit (SK-4800, Vector Laboratories Inc.). Immunostaining for macrophage content was performed as previously described (Cyrus *et al*., 2006; 2007). The avidin-biotin-alkaline phosphatase method (Vector Laboratories and Boeringher Mannhein, GmbH), using rat monoclonal antibody to mouse macrophages (MOMA-2; Accurate Chem. Sci. Corp.) diluted in PBS 1:30, was used. Cross-sections were counterstained with hematoxylin. As control, no primary antibody was added to the same sections. Macrophages, smooth muscle cells and collagen-positive regions were quantified in sections by determination of the area that stained positive for the respective markers (Cyrus *et al*., 2002; 2007). Images of these sections were always captured and analysed without knowledge of the treatments.

Immunofluorescence

Standard immunofluorescence techniques were applied on 8 μm fresh-frozen sections. Briefly, the sections were air-dried, fixed in acetone at 4°C for 10 min, rehydrated with PBS and blocked in 5% bovine serum albumin (BSA) plus TPBS (PBS plus 0.1% Tween-20) at room temperature for 1 h. Primary antibodies (in 5% BSA plus TPBS) were applied at 4°C overnight; after three washes in TPBS, the secondary antibodies (in 5% BSA plus TPBS) were applied at reverse transcriptase (RT) for 30 min. After two more washes in TPBS, 4,6-diamidino-2-phenylindole (DAPI) (0.5 $\mu g \cdot mL^{-1}$, in PBS) was applied for 30 sec before the slides were mounted. Besides, the primary antibody against the receptor for sirolimus [FK506-binding protein (FKBP12); Santa Cruz Biotechnology), a set of co-localization antibody markers were also used. Briefly, macrophages were examined using anti-CD68 antibody (FA-11, Serotec Ltd., Kidlington, UK), FITC-α-actin for smooth muscle cell (Sigma) and DNA fluorescence using DAPI. The following secondary antibodies were used: donkey anti-rabbit IgG, Cy3 conjugated; donkey anti-rat IgG, Cy3 conjugated (Jackson Immunoresearch Laboratories). Antibodies pre-adsorbed with the respective blocking protein were used as negative control.

Reverse transcription and quantitative RT-polymerase chain reaction (PCR)

Total RNA was obtained from snap-frozen aortic tissue using the 'RNeasy mini kit' from QIAGEN (Valencia, CA, USA). DNase digestion was carried out by treating the RNA with the Amplification Grade DNase 1 (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instruction. In order to remove residual DNase and EDTA from the treated RNA, the solution was passed through the RNeasy system for a second time using the RNA clean-up protocol. For monocyte chemoattractant protein-1 (MCP-1) the primers used were 5′-ACTGAAGCCAGCTCTCTCTTCCTC-3′ sense and 5′-TTCC TTCTTGGGGTCAGCACAGAC-3′ antisense; for interleukin-6 (IL-6): 5′-GAAAACAATCTGAAACTTCCA-3′ sense and 5′-TT TTAGAAATTCTTCAAGTGATTTCAAGAT-3′ antisense; for tumour necrosis factor α (TNF-α): 5′-GGCAGGTCTACT TTGGAGTCATTGC-3′ sense and 5′-ACATTCGAGGTCCAG TGAATTCGG-3′ antisense; for interferon γ (IFN-γ): 5′-GCT CTGAGACAATGAACGCT-3′ sense and 5′-AAAGAGATAATC TGGCTCTTGC-3′ antisense; for CD40 were: 5′-TGTGTTAC GTGCAGTGACAAACAG-3′ sense and 5′-GCTTCCTGGCTGG CACAA-3′ antisense; for GAPDH were: 5′-GTCATCATCTCC GCCCCTTCTGC-3′ sense and 5′-GATGCCTGCTTCACCAC CTTCTTG-3′ antisense. Controls consisting of H_2O or samples that were not reversely transcribed were negative for target and reference.

First strand cDNA was synthesized from RNA using the 'Superscript II Reverse Transcriptase' (invitrogen), according to the manufacturer's instructions. The cDNA was used as template for amplification in real-time PCR. Real-time PCR was performed using the ABI PRISM 7900HT sequence Detection System (Applied Bioscience, Foster City, CA, USA). The PCR reaction was performed using a TaqMan gene expression assay consisting of a 20x mix of different unlabelled primers and TaqMan MGB probe FAM ™ (6-carboxylfluorescein) dye-labelled, as previously described (Chinnici *et al*., 2007). Briefly, we also quantitated transcripts of the 18s rRNA gene (Applied Biosystems) with FAM (assay ID Hs99999901_s1) as the internal control, with each unknown sample normalized to 18s rRNA content. We used a TaqMan Universal PCR Master Mix reaction component for a single 20 μL reaction, utilizing the universal thermal cycling parameters.

Statistics

Statistical analyses were performed with the program Graph Pad Prism 4.0. Results were expressed as mean ± standard error of means. All the data were analysed by one-way ANOVA and subsequently by Bonferroni multiple comparisons test. Results that showed $P < 0.05$ were considered significantly different.

Results

Study I: 8-week sirolimus treatment

Starting at 8 weeks of age, LDL r-KO mice were fed a high-fat diet for the entire study. Body weight and total plasma lipids levels were not different among animals when randomized to the four groups ($n = 15$ mice per group) at the beginning of the study (not shown). As expected, animals on placebo achieved a significant increase in weight, plasma cholesterol and triglycerides levels (Table 1). As shown in Figure 1A, immunofluorescence analysis demonstrated abundant positive immunoreactivity for FKBP12, the receptor for sirolimus (Marks, 2003), in the aortic tissues of the mice after 8 weeks on the high-fat diet (Figure S1).

Groups of 15 LDL r-KO mice each (all males) were randomized to receive sirolimus in their diet at 0.1, 0.3 or 1 $mg \cdot kg^{-1}$ for 8 weeks respectively. Compared with placebo, these concentrations of the drug had no effect on weight, plasma

Table 1 Effect of 8-week oral sirolimus administration to LDL r-KO mice on weight, plasma cholesterol, triglycerides, plasma sirolimus and urinary creatinine levels

	Basal	*Placebo*	*Sirolimus (0.1 mg·kg^{-1})*	*Sirolimus (0.3 mg·kg^{-1})*	*Sirolimus (1 mg·kg^{-1})*
Weight (g)	22.0 ± 1.2	34.4 ± 0.9	34.6 ± 0.5	32.2 ± 1.3	33.4 ± 0.4
Cholesterol (mmol·L^{-1})	3.7 ± 0.2	17.6 ± 0.7	20.7 ± 0.5	19.1 ± 0.4	20.5 ± 0.2
Triglycerides (mmol·L^{-1})	1.1 ± 0.1	3.5 ± 0.4	2.9 ± 0.3	4.1 ± 0.5	4.0 ± 0.5
Sirolimus (ng·mL^{-1})	ND	ND	0.19 ± 0.10	0.36 ± 0.11	0.68 ± 0.12
Creatinine (mmol·L^{-1})	3.3 ± 0.3	3.2 ± 0.2	3.4 ± 0.4	3.7 ± 0.3	3.1 ± 0.4

Results are mean ± SEM.
LDL r-KO, low-density lipoprotein receptor-deficient; ND, not detectable.

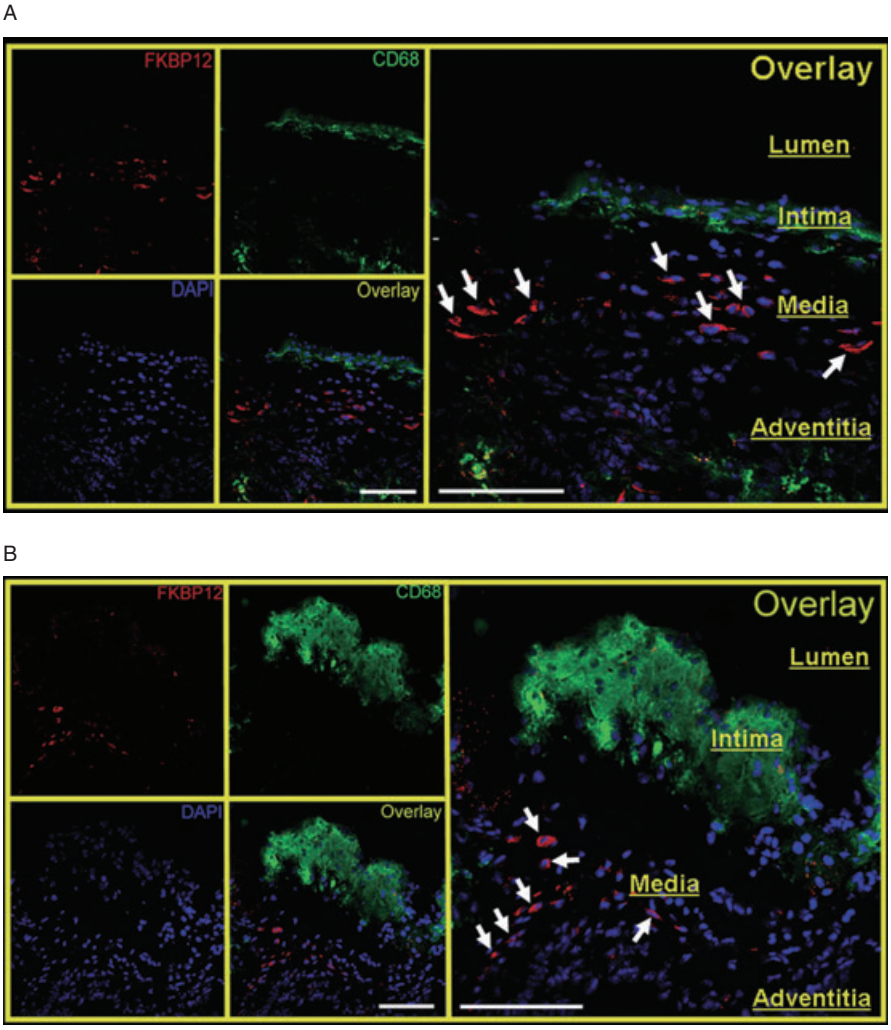

Figure 1 Aortic expression of FKBP12, detected by immunofluorescence, in LDL r-KO mice after 8 (A) and 16 (B) weeks on a high-fat diet. Fresh-frozen aortic sections were air-dried, fixed in acetone at 4°C for 10 min, rehydrated with PBS and blocked in 5% BSA plus TPBS (PBS plus 0.1% Tween-20) at room temperature for 1 h. Primary antibodies used were: anti-FKBP12, anti-CD68 (for macrophages), FITC-α-actin (for smooth muscle cell) and DAPI (for DNA). White arrows indicate positive reaction for FKBP12. Scale bars = 10 μm. DAPI, 4,6-diamidino-2-phenylindole; FKBP12, FK506-binding protein; LDL r-KO, low-density lipoprotein receptor-deficient; PBS, phosphate-buffered saline; TPBS, PBS plus 0.1% Tween-20.

cholesterol and triglyceride levels (Table 1). Compliance with the drug was confirmed by a dose-dependent increase in plasma sirolimus levels in the animals receiving the active drug (Table 1). No sign of nephrotoxicity was observed in the animals receiving the active drug as the levels of urinary creatinine excretion did not significantly differ from the placebo group (Table 1). At the end of the 8-week treatment and 24 h before killing, urine samples were collected and assayed for isoprostane $F_{2\alpha}$-III, a marker of oxidative stress, 2,3dinor TxB_2, a marker of platelet activation, and 2,3dinor 6-oxo-$PGF_{1\alpha}$, a marker of endothelium activation. Compared with placebo, none of the three doses of sirolimus significantly altered the excretion of these markers (data not shown).

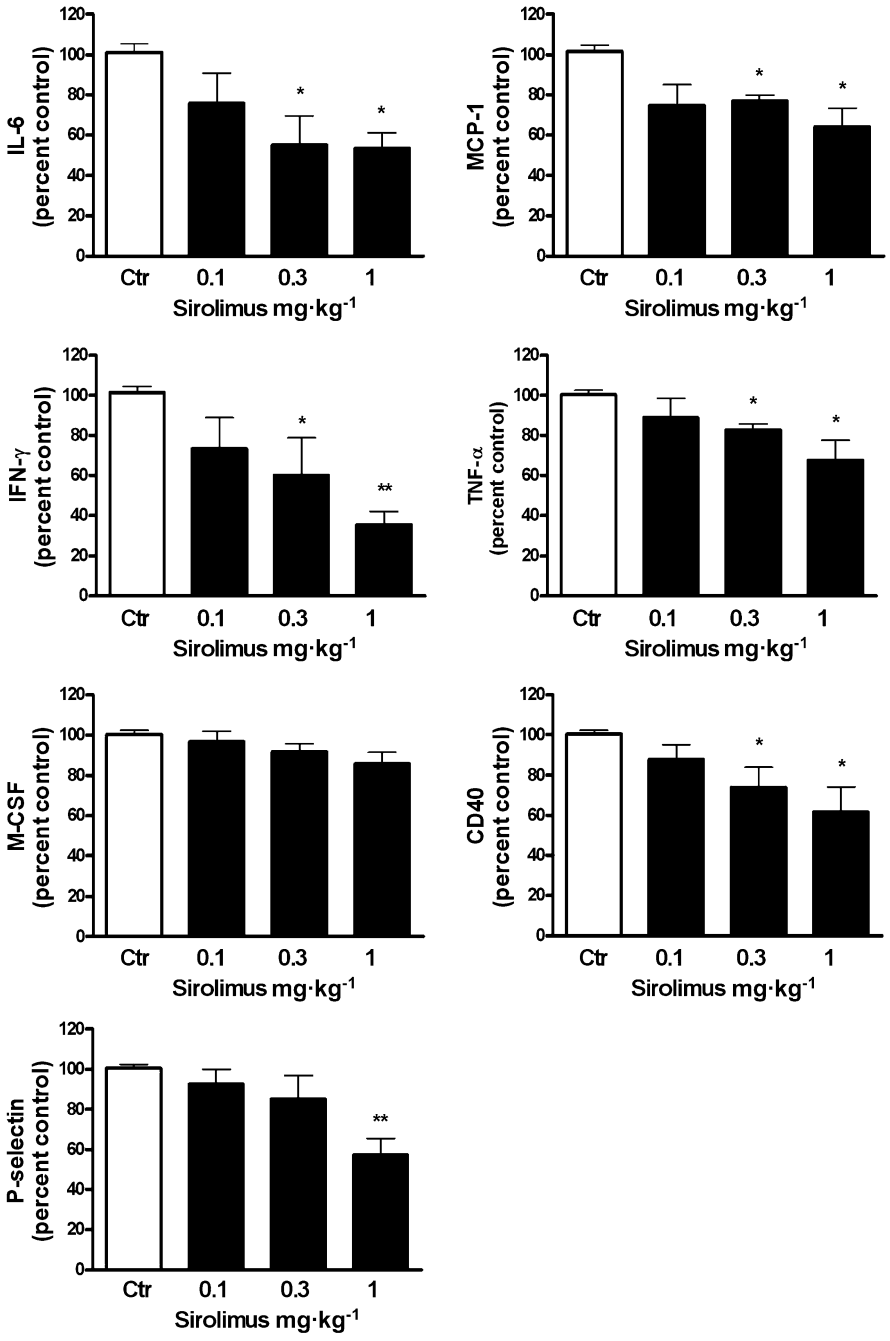

Figure 2 Effect of 8-week sirolimus oral administration on plasma levels of inflammatory cytokines. Cytokine profiles of plasma from LDL r-KO on placebo or treated with the three different doses of the drug. Results for IL-6, MCP-1, IFN-γ, TNF-α, M-CSF, CD40 and P-selectin are shown as mean ± SEM (*$P < 0.05$; **$P < 0.01$). IFN-γ, interferon γ; IL-6, interleukin-6; LDL r-KO, low-density lipoprotein receptor-deficient; M-CSF, macrophage colony stimulating factor; MCP-1, monocyte chemoattractant protein-1; TNF-α, tumour necrosis factor α.

Because atherosclerosis has an important inflammatory component and several inflammatory mediators have been implicated in its pathogenesis, we next examined plasma levels of several pro-atherogenic cytokines (Braunersreuther *et al.*, 2007) by quantitative array analysis. As shown in Figure 2, we observed that among the different cytokines assayed, IL-6, MCP-1, IFN-γ, TNF-α and CD40 were significantly reduced in LDL r-KO mice after treatment with sirolimus. By contrast, while no significant change was observed for macrophage colony stimulating factor (M-CSF) at any sirolimus concentration, we found that P-selectin was significantly reduced only in animals receiving the highest dose (1 mg·kg^{-1}) (Figure 2). Further, sirolimus at 1 mg·kg^{-1} significantly inhibited mRNA levels of MCP-1 (<34%), IL-6 (<35%), TNF-α (<31%) and CD40 (<30%) ($P < 0.05$, for all). Levels of IFN-γ mRNA were always too low in both groups of mice (treated and untreated) for a comparative analysis.

Mice were killed at the end of the 8-week treatment and their aortas analysed for the extent and size of atherosclerotic lesions by two independent methods, the 'en face' method of

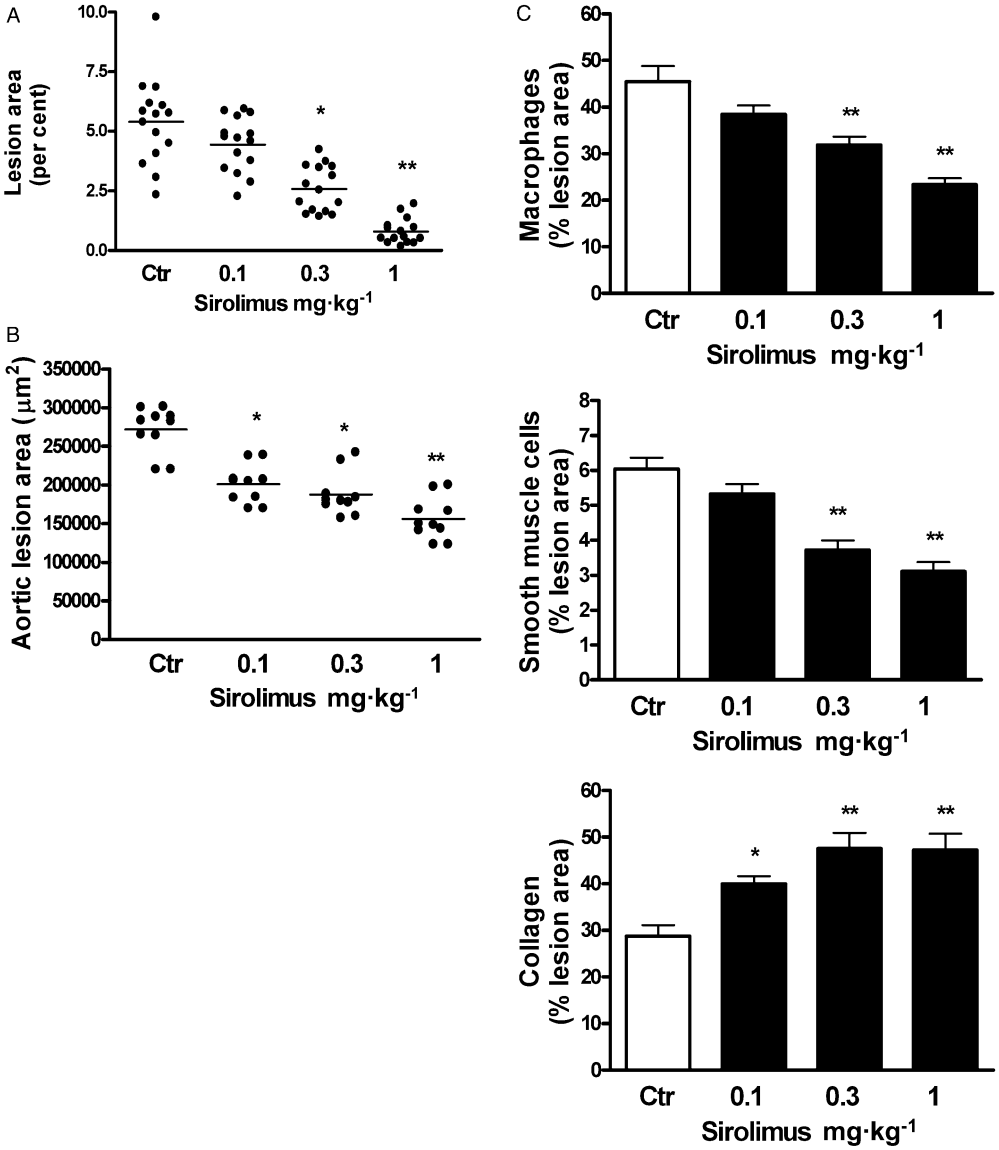

Figure 3 Effect of sirolimus on aortic atherosclerotic lesion development and cellular composition in LDL r-KO mice receiving placebo or three different doses of the drug in their diet for 8-week treatment. (A) Percentage of aortic atherosclerotic lesion area as determined by the 'en face' method. (B) Quantitation of lesion areas of aortic cross-sections. Results are mean ± SEM (*$P < 0.001$; **$P < 0.0001$). (C) Percentage of aortic root atherosclerotic lesions occupied by macrophages (top panel), smooth muscle cells (medium panel) and collagen (bottom panel). Results are mean ± SEM (*$P < 0.05$; **$P < 0.001$). LDL r-KO, low-density lipoprotein receptor-deficient.

the entire aortic tree and cross-section analysis of the proximal aorta.

As expected, after 8 weeks on a high-fat diet, LDL r-KO mice on placebo developed discrete aortic atherosclerotic lesions. However, we found that sirolimus dose-dependently and significantly reduced the atherosclerotic lesion areas in the aortas of the treated animals (Figure 3A). Consistently, cross-section analysis of the aortic sinuses of mice on placebo showed discrete lesion areas after 8 weeks on the high-fat diet. LDL r-KO mice treated with sirolimus had a significant and dose-dependent reduction of the atherosclerotic area when compared with the placebo group (Figure 3B and Figure S2).

Immunocytochemical analyses of cross-sections of the proximal aorta were carried out to investigate whether any of these doses had an impact on plaque composition. Compared with placebo, mice receiving sirolimus showed a dose-dependent decrease in the number of monocytes/macrophages as well as smooth muscle cells in their aortic atherosclerotic lesions (Figure 3C). By contrast, we observed that they manifested a significant increase in the collagen content within the lesions (Figure 3C). No immunostaining was detected in sections where the primary antibody was omitted (data not shown).

Study II: 16-week sirolimus treatment

The same protocol as for study I was followed for study II, except that the period of oral sirolimus treatment was 16 weeks. Body weight and total plasma lipid levels were not different among animals when randomized to the four groups

Table 2 Effect of 16-week oral sirolimus administration to LDL r-KO mice on weight, plasma cholesterol, triglycerides, plasma sirolimus and urinary levels of isoprostane $iPF_{2\alpha}$-III, 2,3dinor TxB_2 and creatinine

	Baseline	*Placebo*	*Sirolimus (0.1 mg·kg^{-1})*	*Sirolimus (0.3 mg·kg^{-1})*	*Sirolimus (1 mg·kg^{-1})*
Weight (g)	22.4 ± 1.1	48.6 ± 0.2	50.4 ± 0.8	49.8 ± 0.7	47.8 ± 0.9
Cholesterol (mmol·L^{-1})	4.0 ± 0.2	25.3 ± 0.3	26.6 ± 0.6	24.7 ± 0.5	23.5 ± 0.7
Triglycerides (mmol·L^{-1})	1.0 ± 0.1	6.9 ± 0.4	6.0 ± 0.5	6.1 ± 0.4	5.8 ± 0.3
Sirolimus (ng·mL^{-1})	ND	ND	0.15 ± 0.05	0.25 ± 0.05	0.65 ± 0.10
$iPF_{2\alpha}$-III (pg·mg·creatinine^{-1})	1.5 ± 0.1	5.6 ± 0.6	6.3 ± 1.1	5.9 ± 0.8	6.3 ± 0.8
2,3dinor TxB_2 (ng·mg·creatinine^{-1})	12 ± 1	83.3 ± 4	92 ± 6	94 ± 11	69 ± 8
Creatinine (mmol·L^{-1})	3.5 ± 0.3	3.6 ± 0.4	3.7 ± 0.5	3.3 ± 0.2	3.9 ± 0.5

Results are mean ± SEM.
2,3dinor TxB_2, 2,3 dinor thromboxane B_2; LDL r-KO, low-density lipoprotein receptor-deficient; ND, not detectable.

($n = 15$ mice per group) at the beginning of the study (not shown). By the end of the study, animals on placebo achieved a significant increase in weight, plasma total cholesterol and triglycerides levels, and urinary excretion of the isoprostane $F_{2\alpha}$-III, 2,3dinor TxB_2, and 2,3dinor 6-oxo-$PGF_{1\alpha}$ (Table 2). Immunofluorescence analysis of the aorta confirmed abundant positive reactivity for FKBP12, the receptor for sirolimus (Marks, 2003), after 16 weeks on the high-fat diet, which was predominantly expressed in the lamina media, but also in the intima and adventitia (Figure 1B and Figure S1).

Groups of 15 LDL r-KO mice each (all males) were randomized to receive sirolimus in their diet at 0.1, 0.3 or 1 mg·kg^{-1} for 16 weeks respectively. Compared with placebo, these concentrations of the drug had no effect on weight, plasma cholesterol and triglycerides levels (Table 2). No sign of nephrotoxicity was observed in the animals receiving the active drug as the levels of urinary creatinine excretion did not differ from the placebo group (Table 2).

By contrast, we observed a dose-dependent increase in plasma sirolimus levels in the animals receiving the active drug (Table 2). At the end of the 16-week treatment and 24 h before killing , urine samples were collected and assayed for in vivo markers of oxidative stress, isoprostane $F_{2\alpha}$-III, platelet activation, 2,3dinor TxB_2, and endothelium activation, 2,3dinor 6-oxo-$PGF_{1\alpha}$. As shown in Table 2, compared with placebo, none of the three doses of sirolimus significantly altered the excretion of isoprostane $F_{2\alpha}$-III, or 2,3dinor TxB_2. In contrast, we observed that mice receiving the medium- and high-sirolimus doses had a significant increase in the urinary excretion of 2,3dinor 6-oxo-$PGF_{1\alpha}$, the stable urinary metabolite of prostacyclin (Figure 4).

Next, as shown in Figure 5, circulating plasma levels of IL-6, MCP-1, IFN-γ, TNF-α and CD40 were significantly and dose-dependently reduced by the end of the study. However, 16 weeks of sirolimus resulted in greater reductions for MCP-1, TNF-α and CD40 than when the drug was administered for only 8 weeks ($P < 0.05$). Again, no significant change was observed for M-CSF at any sirolimus concentration, whereas we found that P-selectin was significantly reduced also in animals receiving the intermediate sirolimus dose (0.3 mg·kg^{-1}) (Figure 5). Interestingly, we also observed that sirolimus at 1 mg·kg^{-1} significantly inhibited mRNA levels of IL-6 (32%), MCP-1 (38%), TNF-α (35%) and CD40 (33%) ($P < 0.05$).

At the end of the treatment, mice were sacrificed and their aortas analysed for the extent of atherosclerotic lesion areas.

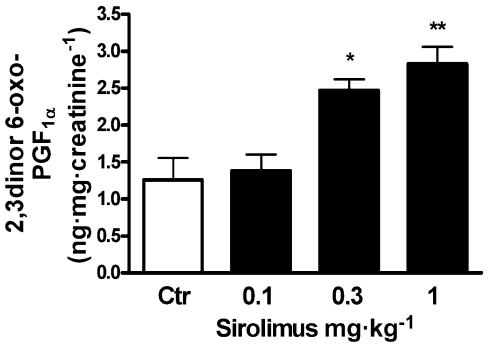

Figure 4 Effect of 16-week oral sirolimus administration to LDL r-KO mice on urinary levels of 2,3dinor 6-oxo-$PGF_{1\alpha}$. Values are shown as mean ± SEM (*$P < 0.01$, **$P < 0.001$). LDL r-KO, low-density lipoprotein receptor-deficient.

As expected, after 16 weeks on a high-fat diet, LDL r-KO mice on placebo developed larger aortic atherosclerotic lesion areas than the mice from study I (5.4 ± 0.4% vs. 19.2 ± 0.8%). Nonetheless, we found that sirolimus dose-dependently and significantly reduced the atherosclerotic lesion areas in the aortas from treated animals (Figure 6A). Interestingly, in contrast to the results with the 8-week treatment with sirolimus, we found that the longer exposure to the drug significantly reduced lesion areas (27% vs. 8%, $P < 0.04$) also in the animals receiving the lowest dose (0.1 mg·kg^{-1}) (Figure 6A). However, no significant difference between 8- and 16-week treatment was observed for the higher doses.

Compared with mice on placebo for 8 weeks, cross-section analysis of the aortic sinuses showed larger lesion areas after 16 weeks on the high-fat diet (271 589 ± 9384 μm^2 vs. 665 680 ± 31 722 μm^2). However, LDL r-KO mice treated with sirolimus had a significant and dose-dependent reduction of the atherosclerotic area when compared with the placebo group (Figure 6B). There was no significant difference in the anti-atherogenic effect between 8- and 16-week treatment for the 0.1 and 0.3 mg·kg^{-1} doses (16% vs. 26% and 40% vs. 34%). By contrast, the effect of 1 mg·kg^{-1} sirolimus after 16 weeks was significantly higher than the effect observed after 8 weeks (62% vs. 38%) ($P < 0.02$).

Immunocytochemical analyses of aortic cross-sections showed that, compared with placebo, mice receiving sirolimus had a dose-dependent decrease in the number of monocytes/macrophages as well as smooth muscle cells in their aortic atherosclerotic lesions (Figure 6C). Interestingly,

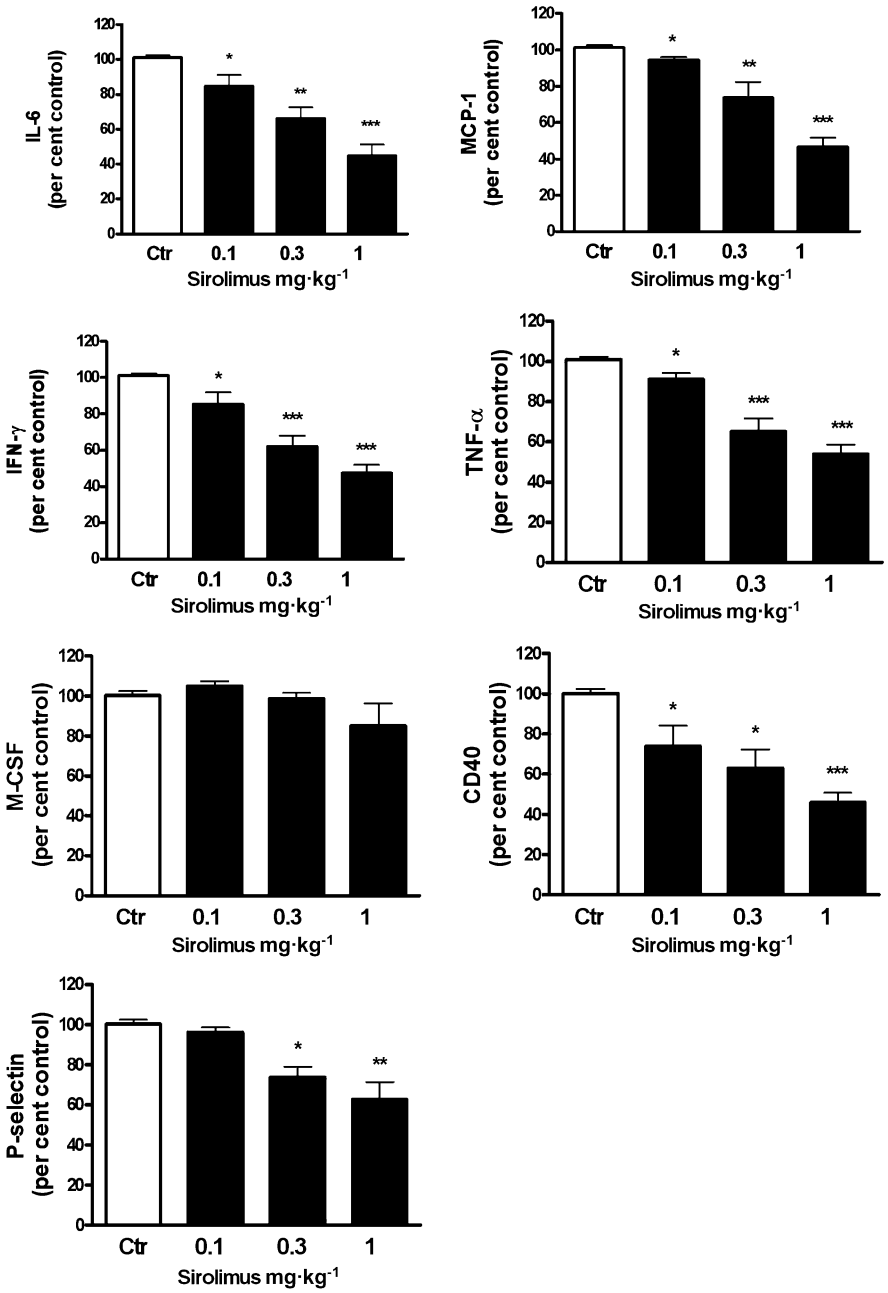

Figure 5 Effect of 16-week oral sirolimus administration on plasma levels of inflammatory cytokines. Cytokine profiles of plasma from LDL r-KO on placebo or treated with the three different doses of the drug. Shown are results for IL-6, MCP-1, IFN-γ, TNF-α, M-CSF, CD40 and P-selectin. Results are mean ± SEM (*$P < 0.05$; **$P < 0.01$; ***$P < 0.001$). IFN-γ, interferon γ; IL-6, interleukin-6; LDL r-KO, low-density lipoprotein receptor-deficient; M-CSF, macrophage colony stimulating factor; MCP-1, monocyte chemoattractant protein-1; TNF-α, tumour necrosis factor α.

we observed that 16-week exposure to sirolimus resulted in a greater reduction in cellularity than that after 8 weeks of treatment. Further, we observed that vessels at 16 weeks manifested a significant and dose-dependent increase in collagen content within the lesions (Figure 6C and Figure S3).

Discussion

In the present study, we demonstrate that low-dose oral sirolimus administration was well tolerated by LDL r-KO mice on a high-fat diet, and that this regimen did not have any significant impact on their body weight, total plasma cholesterol or triglyceride levels. Further, sirolimus dose-dependently and significantly reduced atherogenesis at the aortic roots level and in the entire aortic tree by inducing a strong vascular anti-inflammatory effect, which resulted in a more stable cellular plaque phenotype.

Atherosclerosis is a multifactorial vascular disease in which both lipid metabolism and chronic inflammatory responses are crucial contributing factors. Activation of immune cells, excessive proliferative responses by vascular smooth muscle

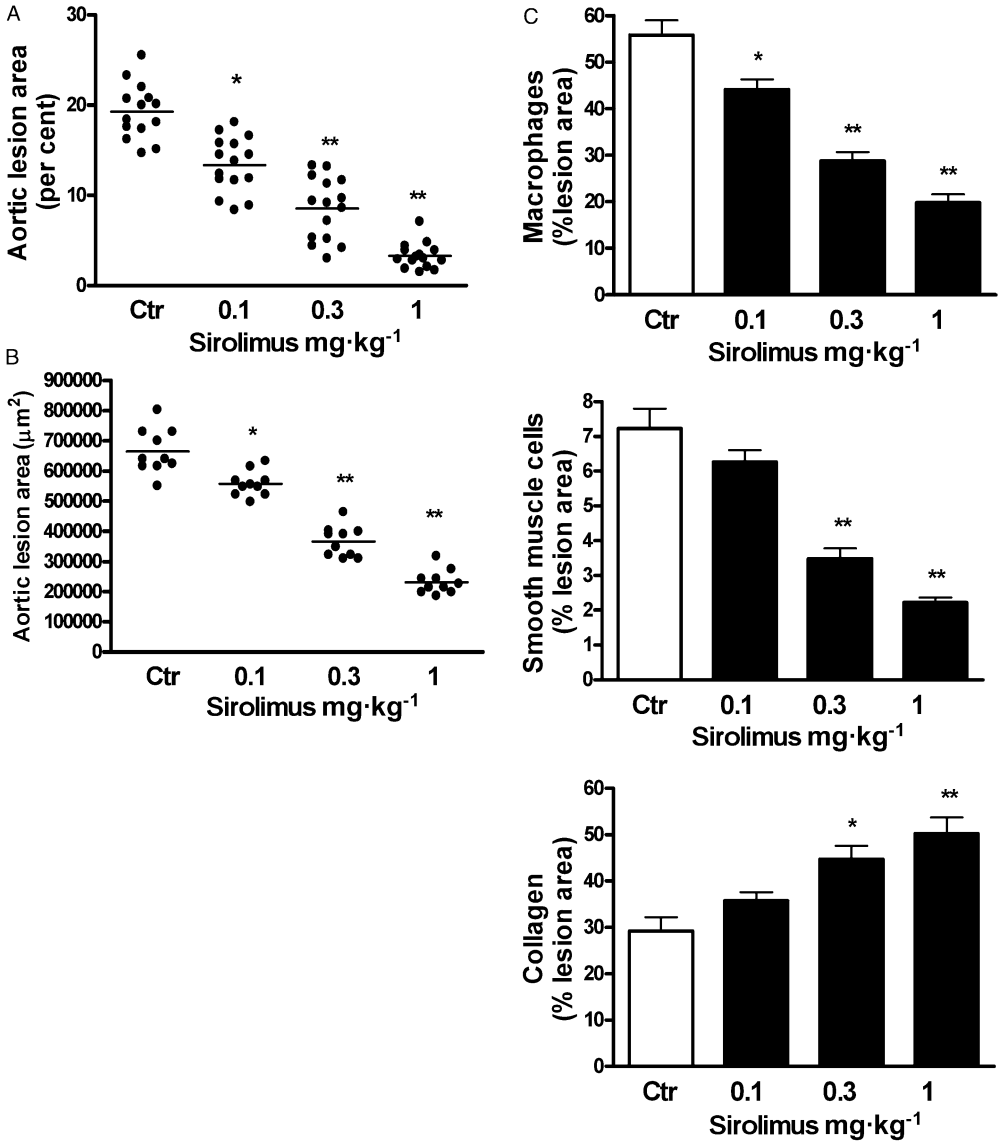

Figure 6 Effect of oral sirolimus administration on aortic atherosclerotic lesion development and cellular composition in LDL r-KO mice after 16-week treatment. (A) Quantitation of atherosclerosis, expressed as percentage of aortic atherosclerotic lesion area, by the 'en face' method. (B) Quantitation of lesion areas of aortic cross-sections. Results are mean ± SEM (*$P < 0.001$; **$P < 0.0001$). (C) Percentage of aortic root atherosclerotic lesions occupied by macrophages (top panel), smooth muscle cells (medium panel) and collagen (bottom panel). Results are mean ± SEM (*$P < 0.01$; **$P < 0.001$). LDL r-KO, low-density lipoprotein receptor-deficient.

cells and monocyte/macrophages and their dysregulated migration have also attracted a lot of attention (Oude Nijhuis *et al*., 2007). However, their functional role in atherosclerotic plaque formation in both humans and experimental animal models remains to be fully established.

Sirolimus is a macrocyclic lactone originally developed as an antibiotic, which manifests its anti-proliferative and anti-migratory actions by binding to the cytosolic FK506-binding protein, known also as FKBP12, a member of the immunophilin family, thereby inhibiting the kinase activity of the protein, known as mammalian target of rapamycin (mTOR) (Sehgal *et al*., 1994; Marks, 2003). It has also been shown that sirolimus attenuates neointimal thickening in experimental models of vascular injury, by directly inhibiting the proliferative and migratory capacities of smooth muscle cells, which are also known to contribute to the atherogenesis (Degertekin *et al*., 2002; Castro *et al*., 2004; Poon *et al*., 2005). Sirolimus was previously found to reduce aortic cholesterol content in apoE-KO mice on a high-fat diet when given intraperitoneally in high doses (>1 mg·kg^{-1}) (Elloso *et al*., 2003; Castro *et al*., 2004). However, despite this local positive effect, it exacerbated pre-existing hypercholesterolemia and hypertriglyceridemia raising concerns about its possible clinical use in atherosclerosis. Thus, some studies in humans showed that at this high-dosage sirolimus indeed could elevate plasma lipids and lipoprotein levels in renal transplantation patients (Hoogeveen *et al*., 2001; Morriset *et al*., 2002; Morales, 2005).

Taken together, these aspects have impaired our ability to evaluate fully the effects of this drug on atherogenesis, without the confounding side-effects. To this end, we wanted

to establish whether or not sirolimus, when used at much lower doses that would not affect plasma lipid levels, would still modulate cell proliferative and inflammatory reactions within the vasculature. Another novel aspect of our studies is the fact that, by contrast with previous work, we administered the drug via the oral route. This route of administration is very important and clinically relevant in view of the potential use of sirolimus as an anti-atherogenic drug.

First, we provided the first evidence of the vascular expression of the sirolimus receptor FKBP12 within the aortic tree of atherosclerotic mice. We showed that this receptor is strongly and predominantly expressed in the lamina media, while sparse and scattered immunoreactivity is also observed in the intima and adventitia. Our results with FKBP12 immunolabeling confirm and extend previous work showing the presence of this receptor in human atheroma as well as in rat carotid arteries after balloon injury (Zohlnhofer *et al*., 2001; Bauriedel *et al*., 2008).

We also demonstrated that the drug at low doses was well tolerated by animals because by the end of the treatment they gained weight regularly, and did not manifest any change in blood cell count, or macroscopic signs of pathology. Finally, compliance with the drug regimen was always confirmed by the dose-dependent increase in the circulating plasma levels of sirolimus.

Human and animal studies have clearly indicated that local production of pro-inflammatory chemokines within the atherosclerotic plaque plays a critical and functional role in the development of atherosclerosis (Libby, 2002; Oude Nijhuis, 2007). Among them, MCP-1, IL-6, TNF-α and CD40 have all been shown to have a pro-atherogenic effect *in vivo* (Tedgui and Mallat, 2006). In our study, we demonstrated for the first time that *in vivo* sirolimus has a potent and selective effect in down-regulating levels of some of the key mediators of the inflammatory vascular phenotype. Thus, the selective and significant reduction in IL-6, MCP-1, TNF-α and CD40 plasma levels was also confirmed by quantitative real-time PCR analysis of the corresponding mRNA levels in aortic tissue.

Intriguingly, we observed that in the 16-week study another potent anti-inflammatory mediator was also increased. Thus, the urinary levels of 6-oxo-$PGF_{1\alpha}$, a measure of total prostacyclin biosynthesis *in vivo* (Dogne *et al*., 2005), was significantly elevated, further supporting the shift of the vascular phenotype towards an anti-atherogenic one in the sirolimus-treated mice (Praticò and Dogne, 2005). As the vasculature is the main source *in vivo* for prostacyclin, it is possible that this increase reflects also the modulatory effect of sirolimus on the cellular components of the vasculature.

It is known that smooth muscle cells in atherosclerotic lesions exhibit a proliferative, de-differentiated and migratory phenotype, which along with the accumulation of macrophage and foam cells, ultimately contributes to athreosclerotic plaque formation (Cizek *et al*., 2007). Confirming previous *in vitro* studies, we observed that both cell types were significantly reduced in the vasculature of mice receiving sirolimus (Ma *et al*., 2007). An interesting recent report showed that the sirolimus derivative, everolimus, reduced macrophages, but not smooth muscle cells, within the atherosclerotic lesion by autophagy (Verheye *et al*., 2007). It is possible that this mechanism is, in part, also involved in our study.

In contrast to the *in vitro* observations, we found that the amount of total collagen within the atherosclerotic lesions was significantly increased in mice receiving sirolimus. It is known that collagen content in the vasculature is the result of a balance between its production by smooth muscle cells and degradation by different proteases (Johnson 2007). As sirolimus has been shown to reduce protein synthesis in smooth muscle cells (Martin *et al*., 2004), we hypothesize that the observed effect on this important marker of lesion stability derives from either decreased catabolism by a reduced expression of metalloproteases, or an increase in their inhibitors, that is, tissue inhibitor of metal proteinase (TIMP). Future studies will determine whether sirolimus can also influence *in vivo* the levels and/or activity of any of these important regulatory mechanisms of collagen synthesis.

A potential limitation of our study is that the pharmacokinetics of sirolimus in mice could be different from that in humans and that, whereas humans would get a once or twice-daily dose, in our studies mice were continuously dosed with the drug by receiving it in their food. These aspects need to be considered before extrapolating any results from our studies to humans.

In summary, our results demonstrate for the first time that low-dose oral sirolimus is well tolerated, and exerts a potent anti-atherosclerotic action *in vivo* in the LDL r-KO mice, without influencing total lipid levels. We provide evidence that this activity is mediated by a selective vascular anti-inflammatory effect, which ultimately results in a more stable atherosclerotic lesion phenotype characterized by a reduction in smooth muscle cell proliferation and an increase in collagen content within the atheroma. We conclude that low-dose oral sirolimus could represent a novel pharmacological strategy aimed at preventing or delaying the progression of human atherosclerosis.

Acknowledgement

We thank Yutian Zhan for technical assistance with the immunohistochemistry studies of FKBP12. This study was supported by a grant from Cordis Corporation, a Johnson & Johnson Company, Warren, NJ.

Conflict of interests

Dr Falotico is a current employee and stockholder of Cordis Corporation. At the time of this study, Dr Zhao was an employee of Cordis Corporation.

References

Bauriedel G, Jabs A, Kraemer S, Nickenig G, Skowasch D (2008). Neointimal expression of rapamycin receptor FK506-binding protein FKBP12: post-injury animal and human in-stent restenosis tissue characteristics. *J Vasc Res* **45**: 173–178.

Braunersreuther V, Mach F, Steffens S (2007). The specific role of chemokines in atherosclerosis. *Thromb Haemost* **97**: 714–721.

Castro C, Campistol JM, Sancho D, Sanchez-Madrid F, Casals E, Andres V (2004). Rapamycin attenuates atherosclerosis induced by dietary cholesterol in apolipoprotein E-deficient mice through a p27 Kip1-independent pathway. *Atherosclerosis* **172**: 31–38.

Chinnici C, Yao Y, Praticò D (2007). The 5-lipoxygenase enzymatic pathway in the mouse brain: young versus old. *Neurobiol Aging* **28**: 1457–1462.

Cizek SM, Bedri S, Talusan P, Silva N, Lee H, Stone JR (2007). Risk factors for atherosclerosis and the development of preatherosclerotic intimal hyperplasia. *Cardiovasc Pathol* **16**: 344–350.

Cyrus T, Sung S, Zhao L, Funk CD, Tang S, Praticò D (2002). Effect of low-doses aspirin on vascular inflammation, plaque stability, and atherogenesis in low density lipoprotein receptor deficient mice. *Circulation* **106**: 182–1287.

Cyrus T, Yao Y, Tung L, Praticò D (2006). Regression of established atherosclerosis in low density lipoprotein deficient mice by low-dose aspirin. *Atherosclerosis* **184**: 8–14.

Cyrus T, Yao Y, Ding T, Dogne JM, Praticò D (2007). A novel thromboxane receptor antagonist and synthase inhibitor, BM573, reduces development and progression of atherosclerosis in LDL receptor deficient mice. *Eur J Pharmacol* **561**: 105–111.

Degertekin M, Regar E, Tanabe K, Lee CH, Serruys PW (2002). Sirolimus eluting stent in the treatment of atherosclerosis coronary artery disease. *Minerva Cardioangiol* **50**: 405–418.

Dogne JM, Hanson J, Praticò D (2005). Thromboxane, prostacyclin and isoprostanes: therapeutic targets in atherogenesis. *Trends Pharmacol Sci* **26**: 639–644.

Elloso MM, Azrolan N, Sehgal SN, Hsu P, Ohiel KL, Kopec CA *et al.* (2003). Protective effect of the immunosuppressant sirolimus against aortic atherosclerosis in Apo E-deficient mice. *Am J Transpl* **3**: 562–569.

Farb A, John M, Acamapado E, Kolodgie FD, Prescott MF, Virmani R (2002). Oral everolimus inhibits in-stent neointimal growth. *Circulation* **106**: 2379–2389.

Gall R, Padurean A, Jayaraman T, Marx S, Roque M, Adelman S *et al.* (1999). Inhibition of intimal thickening after balloon angioplasty in porcine coronary arteries by targeting regulators of the cell cycle. *Circulation* **99**: 2164–2170.

Greaves DR, Channon KM (2002). Inflammation and immune responses in atherosclerosis. *Trends Immunol* **23**: 535–541.

Hansson GK, Libby P, Schonbeck U, Yan ZQ (2003). Innate and adaptive immunity in the pathogenesis of atherosclerosis. *Circ Res* **92**: 281–291.

Hoogeveen RC, Ballantyne CM, Pownall HJ, Opekun AR, Hachey DL, Jaffe JS *et al.* (2001). Effect of sirolimus on the metabolism of ApoB100-containing lipoproteins in renal transplant patients. *Transplantation* **72**: 1244–1250.

Johnson JL (2007). Matrix metalloproteinases: influence on smooth muscle cells and atherosclerotic plaque stability. *Expert Rev Cardiovasc Ther* **5**: 265–282.

Libby P (2002). Inflammation in atherosclerosis. *Nature* **420**: 868–874.

Ma KL, Ruan XZ, Powis SH, Moorhead JF, Vargese Z (2007). Anti-atherosclerotic effect of sirolimus on human vascular smooth muscle cells. *Am J Physiol Heart Circ Physiol* **292**: H2721–H2728.

Marks AR (2003). Rapamycin: signaling in vascular smooth muscle. *Transplant Proc* **35**: 231S–233S.

Martin KA, Rzucidlo EM, Merenick BL, Fingar DC, Brown JD, Wagner RJ *et al.* (2004). The mTOR/p70 S6K1 pathway regulates vascular smooth muscle cell differentiation. *Am J Physiol Cell Physiol* **286**: 507–517.

Morales JM (2005). Cardiovascular risk profile in patients treated with sirolimus after renal transplantation. *Kidney Int* **67**: S69–S73.

Morris RE, Cao W, Huang X, Gregory CR, Billingham ME, Rowan R *et al.* (1995). Rapamycicn (Sirolimus) inhibits vascular smooth muscle DNA synthesis in vitro and suppresses narrowing in arterial allograft and in balloon-injured carotid arteries: evidence that rapamycin antagonizes growth factor action on immune and non-immune cells. *Transplant Proc* **27**: 430–431.

Morriset JD, Abdel-Fattah G, Hoogeveen R, Mitchell E, Ballantyne CM, Pownall HJ *et al.* (2002). Effects of sirolimus on plasma lipids, lipoprotein levels, and fatty acid metabolism in renal transplant patients. *J Lipid Res* **43**: 1170–1180.

Naoum JJ, Woodside KJ, Zhang S, Rychahou PG, Hunter GC (2005). Effects of rapamycicn on the arterial inflammatory response in atherosclerotic plaques in Apo-E knockout mice. *Transpl Proc* **37**: 1880–1884.

Oude Nijhuis MM, van Keulen JK, Pasterkamp G, Quax PH, de Kleijn DP (2007). Activation of the innate immune system in atherosclerotic disease. *Curr Pharm Des* **13**: 983–994.

Poon M, Marx SO, Gallo R, Badimon JJ, Taubman MB, Marks AR (2005). Rapamycin inhibits vascular smooth muscle cell migration. *J Clin Invest* **98**: 2277–2283.

Praticò D, Dogne J-M. (2005). Selective cyclooxygenase-2 inhibitors development in cardiovascular medicine. *Circulation* **112**: 1073–1079.

Sehgal SN, Molnar-Kimber K, Ocain TD, Weichman BM (1994). Rapamycin: a novel immunosuppressive macrolide. *Med Res Rev* **14**: 1–22.

Snell GI, Levvey BJ, Chin W, Kotsimbos T, Whitford H, Waters KN *et al.* (2003). Sirolimus allows renal recovery in lung and heart transplant recipients with chronic renal impairment. *J Heart Lung Transplant* **21**: 540–546.

Sousa JE, Costa MA, Abizaid A, Abizaid AS, Feres F, Pinto IM *et al.* (2001). Lack of neointimal proliferation after implantation of sirolimus-coated stents in human coronary arteries: a quantitative coronary angiography and three-dimentional intravascular ultrasound study. *Circulation* **103**: 192–195.

Tedgui A, Mallat Z (2006). Cytokines in atherosclerosis: pathogenic and regulatory pathways. *Physiol Rev* **86**: 515–581.

Verheye S, Martinet W, Kockx MM, Knaapen MWM, Salu K, Timmermans JP *et al.* (2007). Selective clearance of macrophages in atherosclerotic plaques by autophagy. *J Am Coll Cardiol* **49**: 706–715.

Zohlnhofer D, Klein CA, Richter T, Brandl R, Murr A, Nuhrenberg T *et al.* (2001). Gene expression profiling of human stent-induced neointima by cDNA array analysis of microscopic specimens retrieved by helix cutter atherectomy. Detection of FK506-binding protein 12 upregulation. *Circulation* **103**: 1396–1402.

Supporting Information

Additional Supporting Information may be found in the online version of this article:

Figure S1 Immunostaining of FKBP12 in atherosclerotic lesions. Aortic root sections from LDL-R$^{-/-}$ mice were immunostained with the anti-FKBP12 antibody (ABR) pre-incubated with blocking peptide (A and B, negative controls) and anti-FKBP12 antibody to visualize FKBP12 expression in smooth muscle-rich areas (C and D). Representative of six mice. FKBP12, FK506-binding protein; LDL-R, low-density lipoprotein receptor.

Figure S2 Representative photomicrographs of aortic sinus lesion area of atherosclerotic LDL r-KO mice after 8-week treatment on placebo or sirolimus (0.1; 0.3; 1 mg·kg^{-1}). Sections were immunostained for macrophage content (Mono), smooth muscle cells (SMC) or collagen. LDL r-KO, low-density lipoprotein receptor-deficient.

Figure S3 Representative photomicrographs of aortic sinus lesion area of atherosclerotic LDL r-KO mice after 16-week

treatment on placebo or sirolimus (0.1; 0.3; 1 mg·kg^{-1}). Sections were immunostained for macrophage content (Mono), smooth muscle cells (SMC) or collagen. LDL r-KO, low-density lipoprotein receptor-deficient.