Rapamune Does Not Attenuate High Cholesterol-Induced Atherosclerosis in Rabbits

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Abstract

Keywords

- atherosclerosis
- Rapamune
- serum lipids
- malondialdehyde
- protein carbonyls
- macrophage accumulation
- high cholesterol diet

Solid-organ transplant recipients are prone to develop atherosclerosis. The objectives of this study were to investigate the effects of Rapamune (Wyeth Canada, Saint-Larent, QC, Canada) on the rabbit model of atherosclerosis. The rabbits were assigned to four groups: group I, regular diet (control); group II, 1% cholesterol diet; group III, control with Rapamune (1 mg/kg/d orally); and group IV, high cholesterol diet with Rapamune. Blood samples for serum lipids (triglycerides [TG], total cholesterol [TC], low-density lipoprotein cholesterol [LDL-C], high-density lipoprotein cholesterol [HDL-C]), as well as malondialdehyde, and protein carbonyls, the indices of oxidative stress were collected at the end of 2 months on the respective diet regimen. Aortic tissue for atherosclerotic changes were also collected for oxidative stress indices were also collected. Rapamune reduced serum levels of TG, TC, LDL-C, and HDL-C. Rapamune elevated the oxidative stress in rabbits on high cholesterol diet. Rapamune did not attenuate extent of atherosclerosis (group II vs. group IV, 45.00 ± 12.00 vs. $57.28 \pm 2.99\%$); intimal thickness (group II vs. group IV, $32.38 \pm 7.14 \times 10^3$ vs. $21.90 \pm 11.98 \times 10^3 \mu\text{m}^2$); intimal/medial ratio (group II vs. group IV, 0.50 ± 0.06 vs. 0.35 ± 0.06); and macrophage accumulation (group II vs. group IV, 69.72 ± 5.02 vs. $61.52 \pm 8.94\%$) in the intima of rabbits on high cholesterol diet. The data suggest that (1) Rapamune increased the oxidative stress in rabbits on high cholesterol diet and (2) Rapamune did not attenuate the hypercholesterolemic atherosclerosis in the rabbit model.

Solid-organ transplant recipients are prone to develop atherosclerosis due to multiple factors including immunosuppressive therapy such as cyclosporine A.^{1,2} Rapamune (Wyeth Canada, Saint-Larent, QC, Canada) is a novel immunosuppressive drug capable of significantly reducing the acute graft rejection in solid-organ transplant. Rapamune has direct inhibitory effects on smooth muscle cell proliferation³ and inhibits migration in the intima.⁴ Rapamune eluted stents have been used to prevent postpercutaneous coronary intervention restenosis.⁵

The growing evidence suggests that Rapamune may have paradoxical proinflammatory effects.⁶ There are numerous

risk factors for atherosclerosis including hypercholesterolemia, hypertriglyceridemia, hyperglycemia, and inflammation.^{7–10} Rapamune, because of its antiproliferative, antifibrotic, and anti-inflammatory effect,¹¹ could attenuate atherosclerosis in transplant recipients. However, transplant patients on oral Rapamune still develop atherosclerosis. In genetically modified murine model of atherosclerosis (apolipoprotein E [apo E]-deficient mice) which is not fully comparable to human situation, Rapamune has been shown to attenuate atherosclerosis in porcine models.^{12,13} Rapamune has been shown to sustain neointimal hyperplasia for 6 months after sirolimus eluting stent (SES) implantation.¹⁴

Neointimal formation after balloon-induced vascular injury in Yucatan minipigs is reduced by oral rapamycin.^{12,13} The foregoing data on the effects of Rapamune on atherosclerosis are inadequate and conflicting. Rabbit model of high cholesterol diet-induced atherosclerosis is a good model to study the effects of drugs on atherosclerosis.

An investigation was therefore made of the effects of high cholesterol diet in rabbits with or without Rapamune on the development of atherosclerosis, serum lipids profile (triglycerides [TG], total cholesterol [TC], low-density lipoprotein cholesterol [LDL-C], high-density lipoprotein cholesterol [HDL-C]), and serum and aortic malondialdehyde (MDA), and protein carbonyls, the indices of oxidative stress.

Methods

Drugs

Rapamune sirolimus oral solution (1 mg/mL) was supplied by Wyeth Canada.

Experimental Animals and Study Design

A total of 24 female New Zealand white rabbits weighing between 1.8 and 2.0 kg (6–8 weeks old) were assigned to four groups (► **Table 1**), after 1 week of acclimatization on regular rabbit chow diet. The rabbits in group I were fed regular diet. The other groups received Rapamune without (group III) and with high cholesterol diet (group IV) or high cholesterol diet (group II) in addition to rabbit chow diet. The diet was prepared by Purina (St. Louis, MO) and did not contain any antioxidants. Rapamune was administered through nasogastric tubes. Food and water were allowed ad libitum. The rabbits were housed in individual cages at room temperature of 20 to 22°C with relative humidity of 40 to 70% under a 12-hour light and 12-hour dark cycle. The experimental protocols were approved by the Ethics Committee of the University of Saskatchewan and the animal care was performed according to the approved standards for Laboratory Animal Care. The rabbits were on their respective diet regimen for 2 months. Eighteen hour fasting blood samples (from ear marginal artery) were collected at the end of the protocol for measurement of serum lipids (TG, TC, LDL-C, and HDL-C), MDA, and protein carbonyl contents. At the end of the protocol, the rabbits were anesthetized with Euthanyl (pentobarbital sodium) (50 mg/kg intravenously) (Bimeda-MTC Animal Health Inc., Cambridge, ON, Canada) and aortas were

removed for assessment of atherosclerotic plaques, aortic MDA, and aortic protein carbonyls.

Serum Lipids

Serum TG, TC, LDL-C, and HDL-C were measured on an automated Beckman Synchron LX 20 Clinical System Analyzer, Chemistry, Brea, CA.

Preparation of the Aortic Tissue for Measurement of Malondialdehyde and Protein Carbonyls

The aortas between the origin and bifurcation to iliac arteries were removed and a small aortic ring at the origin was excised and kept in 10% buffered formalin for histological examination. The rest of the aortas were cleansed of gross adventitial tissue and cut longitudinally into two halves, one half was used for assessment of atherosclerotic changes, and the other half was kept in ice-cooled test tubes for preparation of the supernatant by previously described method¹⁵ for measurement of MDA and protein carbonyls.

Serum and Aortic Malondialdehyde

MDA levels of serum and aortic supernatant were measured as thiobarbituric acid reactive substances (TBARs) using previously described method.¹⁶ TBARs were extracted using a mixture of butanol:pyridine (15:1 by volume) and separated by centrifugation. The fluorescence intensity of TBARs in the butanol-pyridine mixture was measured at 553 nm with excitation at 513 nm. The MDA concentration of the tissue was expressed as nmol/mg protein and that of serum as nmol/mL.

Serum and Aortic Protein Carbonyls

The levels of protein carbonyls in the aortic tissue supernatant and serum were measured using a Cayman Chemical Carbonyl Protein Assay Kit (Cayman Chemical Company, Ann Arbor, MI). The absorbance was measured at a wavelength between 360 and 386 nm using an ELx 808TM Absorbance microplate reader (BioTek Instruments, Inc., Highland Park, VT). The protein carbonyls content of the tissue was expressed as nmol/mg proteins and that of serum as nmol/mL.

Tissue Protein Measurement

Protein content of the supernatant of aorta and the serum was determined using a Modified Lowry Protein Assay Kit (Pierce Biotechnology, Rockford, IL). The absorbance was measured at a wavelength of 750 nm using an ELx 808TM Absorbance microplate reader (BioTek Instruments, Inc.).

Assessment of Atherosclerotic Changes in Aorta

Assessment of atherosclerotic changes in the aorta was performed by using Herxheimer solution containing Sudan IV for lipid staining as described previously.¹⁷ Photographs of the stained intimal surface of the aorta were taken with a digital camera. The total and atherosclerotic areas of the intimal surface of the aorta were measured by using image analysis software Scion Image for Windows (Scion Corporation, Frederick, MD). The extent of atherosclerosis was expressed as a percentage of total intimal surface area.

Table 1 Experimental diet groups

Groups	Diet/treatment
I (n = 6)	Control (regular rabbit chow diet)
II (n = 6)	1% cholesterol diet (1% cholesterol in rabbit chow diet)
III (n = 6)	Control + Rapamune (1 mg/kg/d orally)
IV (n = 6)	1% cholesterol + Rapamune (1% cholesterol + 1 mg/kg/d orally)

Table 2 Serum levels of TG, TC, LDL-C, and HDL-C in the four experimental groups

Parameters	Group I	Group II	Group III	Group IV
TG (mmol/L)	0.49 ± 0.30	6.65 ± 1.91 ^a	0.73 ± 0.12 ^b	1.14 ± 0.32 ^b
TC (mmol/L)	1.31 ± 0.33	73.80 ± 4.65 ^a	1.67 ± 0.47 ^b	29.04 ± 9.58 ^{a,b,c}
LDL-C (mmol/L)	0.50 ± 0.20	61.54 ± 3.72 ^a	Not available	25.63 ± 9.21 ^{a,b}
HDL-C (mmol/L)	0.59 ± 0.21	9.25 ± 1.25 ^a	0.78 ± 0.19 ^b	2.9 ± 0.56 ^{a,b,c}

Abbreviations: HDL-C, high-density lipoprotein cholesterol; LDL-C, low-density lipoprotein cholesterol; TC, total cholesterol TG, triglycerides.

Notes: Group I, control; group II, 1% cholesterol diet; group III, control + Rapamune; and group IV, 1% cholesterol + Rapamune. Results are expressed as mean ± standard deviation.

^a*p* < 0.05, control vs. groups II–IV.

^b*p* < 0.05, group II vs. groups III and IV.

^c*p* < 0.05, group III vs. group IV.

Quantification and Histological Assessment of Atherosclerotic Lesions in Aortic Rings

Sections stained with hematoxylin-eosin (H&E) stain and Movat stain were used to measure the total cross-sectional intimal and medial areas (thickness) and the intimal/medial thickness (I/M) ratio. By using image analysis software, Scion Image for Windows (Scion Corporation), the total cross-sectional intimal area was measured between the endothelial cell monolayer and the internal elastic lamina (IEL), and the total cross-sectional medial area was measured between the external elastic lamina and the IEL. The intimal and medial surface areas as well as the I/M ratio were taken as measures of the severity of atherosclerosis.

Assessment of Cytoplasmic Macrophage Expression

The aortic rings specimens were cut across and embedded in paraffin. Serial 4-μm-thick sections cut onto positively charged slides and deparaffinized and subjected to antigen retrieval by microwaving in 10 mM of citrate buffer (sodium citrate, pH 6.0) for 30 minutes. Slides were microwaved in 1 mM EDTA buffer (pH 9.0) for 13 minutes for heat-induced epitope retrieval. Immunohistochemical staining was performed using Dako autostainer and the EnVision plus kits and reagents from DAKO Corporation (Carpinteria, CA). The sections were incubated with antimacrophage (Clone: RAM11) (DAKO Corporation) monoclonal antibody at dilution of 1:1,000. After staining, the Aperio ScanScope CS Slide Scanner (Aperio Technologies, Aperio, CA) system was used to capture whole-slide digital images with a ×20 objective. A positive pixel count algorithm (Aperio Technologies) was used to develop a qualitative scoring model for cytoplasmic macrophage expression.

Statistical Analysis

All statistical analyses were made using Statistical Package for the Social Sciences version 11.0 (SPSS, Inc. Chicago, IL). Results were expressed as mean ± standard deviation. Repeated-measures analysis of variance was used for statistical analysis and Tukey post hoc was used for pairwise multiple comparisons. A *p* value of < 0.05 was considered statistically significant.

Results

Body Weight

The body weight in groups I–IV were 2.92 ± 0.12, 2.95 ± 0.09, 2.83 ± 0.13, and 2.73 ± 0.14 kg, respectively, and they were not significantly different from each other.

Serum Lipids

The values of the TG, TC, LDL-C, and HDL-C of the four experimental groups are summarized in ► **Table 2**. The values of serum TG in groups I, III, and IV were similar but values in group II were higher than those in other groups. The data suggest that Rapamune does not lower the levels of serum TG in normocholesterolemic and hypercholesterolemic rabbits.

Serum TC levels were similar in groups I and III. The values in groups II and IV were higher than those in groups I and III. The TC levels in serum were lower in group IV compared with group II suggesting that Rapamune lowers the serum levels TC in hypercholesterolemic rabbits but has no effect in normocholesterolemic rabbits.

Serum LDL-C levels were lower in group IV compared with group II but these values were higher compared with those in group I, suggesting that Rapamune does lower serum LDL-C levels.

Serum levels of HDL-C were similar in groups I and III but were lower than those in groups II and IV. The values of HDL-C in group IV were lower than those in group II. The data suggest that Rapamune lowers the serum levels of HDL-C in hypercholesterolemic but has no effect in normocholesterolemic rabbits.

Malondialdehyde and Protein Carbonyls in Serum and Aorta

The serum MDA and protein carbonyls of the four groups are summarized in ► **Table 3**. The serum MDA in groups I, III, and IV were similar but lower than those in group II. The levels of serum protein carbonyls in groups II, III, and IV were higher than those in group I. The values in groups III and IV were higher compared with those in group II. The values in group IV were higher than those in group II. The data suggest that Rapamune elevated the oxidative stress in the rabbit with or without high cholesterol diet. The data on aortic MDA and protein carbonyls of the four experimental groups are

Table 3 MDA and protein carbonyls levels of serum and aorta in the four experimental groups

Parameters	Group I	Group II	Group III	Group IV
Serum MDA (mmol/L)	0.58 ± 0.05	18.34 ± 3.08 ^a	0.94 ± 0.28 ^b	3.62 ± 0.45 ^b
Serum protein carbonyls content (nmol/mL)	1.37 ± 0.19	5.34 ± 1.37 ^{a,c}	10.00 ± 0.87 ^{a,b}	10.57 ± 0.84 ^{a,b}
Aorta MDA (nmol/mg)	0.66 ± 0.25	0.53 ± 0.32	1.61 ± 1.36	1.04 ± 0.62
Aorta protein carbonyls content (nmol/mg)	0.40 ± 0.25	0.49 ± 0.39	0.78 ± 0.28	1.92 ± 1.20 ^{a,b}

Abbreviation: MDA, malondialdehyde.

Notes: Group I, control; group II, 1% cholesterol diet; group III, control + Rapamune; and group IV, 1% cholesterol + Rapamune. Results are expressed as mean ± standard deviation.

^a*p* < 0.05, control vs. groups II–IV.

^b*p* < 0.05, group II vs. groups III and IV.

^c*p* < 0.05, group III vs. group IV.

summarized in ►Table 3. The aortic MDA levels were similar in all the groups. The protein carbonyl contents of the aorta in groups I, II, and III were similar but were lower than those in group IV. The data suggest that Rapamune elevated the oxidative stress in rabbits on high cholesterol diet.

Atherosclerotic Changes in the Aorta

The data on the atherosclerotic changes in the aorta of the four groups are summarized in ►Table 4. Atherosclerotic plaques were absent in the aorta from groups I and III. Significant areas of the intimal surface of the aortas from group II (45.00 ± 12.00%) and group IV (57.28 ± 2.99%) were covered with atherosclerotic plaques. The values of the atherosclerotic changes were similar in groups II and IV suggesting that Rapamune did not suppress the development of hypercholesterolemic atherosclerosis.

Histological Evaluation of Atherosclerotic Changes in Aorta

The histological sections of the aorta stained with H&E stain from four groups are shown in ►Figs. 1 and 2. Histological sections through the atherosclerotic plaques of the aorta from groups II and IV showed thickening of the intima which contained foam cells. Histological sections stained with Movat stain showed that the IEL and elastic fibers in the sub-intimal media were intact and arranged in normal fashion

(►Fig. 3). The changes in the intimal and medial area and the ratio of intimal to medial area (I/M) are shown in ►Table 4. The values for intimal areas of groups I and III were similar but were lower compared with those of group II. The intimal areas in groups II and IV were 4.27- and 2.88-fold higher than those in group I. The values in groups II and IV were not significantly different from each other. The values for the medial thickness in group I were similar to those in groups II, III, and IV. However, the values in group III were lower compared with those in groups II and IV. I/M ratio was higher in groups II and IV compared with those in group I. The values were similar in groups II and IV but higher than those in group III.

These data suggest that high cholesterol diet increased the intimal thickness and the I/M ratio but did not alter the medial thickness. Rapamune did not alter the high cholesterol diet-induced changes in the intimal thickness and I/M ratio.

Macrophage Content of the Aortic Intimal Area

Representative photographs of the histological cross section of aortic ring and magnified section of part of the aortic stained with macrophage stain (Clone: RAM11) from the four experimental groups are shown, respectively, in ►Figs. 4 and 5 and the results are summarized in ►Fig. 6. A significant area of the intima of the aorta was covered with macrophages in group II (69.72 ± 5.02%) and group IV (61.52 ± 8.94%). Only a small percentage of the intima was covered with

Table 4 Extent of atherosclerotic changes in the aorta of the four experimental groups

Parameters	Group I	Group II	Group III	Group IV
Atherosclerosis (% of total intimal surface)	0.00	45.00 ± 12.00 ^a	0.00 ^b	57.28 ± 2.99 ^{a,c}
Intimal area (× 10 ³ μm ²)	7.59 ± 1.53	32.38 ± 7.14 ^a	5.50 ± 1.17 ^b	21.90 ± 11.98 ^c
Medial area (× 10 ³ μm ²)	52.31 ± 9.71	64.57 ± 10.88	42.60 ± 5.66 ^b	61.77 ± 12.96 ^c
I/M ratio	0.15 ± 0.01	0.50 ± 0.06 ^a	0.13 ± 0.01 ^b	0.35 ± 0.06 ^{a,c}

Abbreviation: I/M, intimal/medial.

Notes: Group I, control; group II, 1% cholesterol diet; group III, control + Rapamune; and group IV, 1% cholesterol + Rapamune. Results are expressed as mean ± standard deviation.

^a*p* < 0.05, control vs. groups II–IV.

^b*p* < 0.05, group II vs. groups III and IV.

^c*p* < 0.05, group III vs. group IV.

Fig. 1 Histological sections of aorta from four experimental groups of rabbits stained with hematoxylin-eosin showing atherosclerotic changes to measure intima and media thickness. Magnification is shown in the figure. Group I, control; group II, 1% cholesterol diet; group III, control + Rapamune; and group IV, 1% cholesterol + Rapamune.

macrophage in group I ($3.19 \pm 1.58\%$) and group III ($6.81 \pm 1.66\%$). The percentages of intimal area covered with macrophage were greater in groups II and IV compared with groups I and III. The values were similar in groups II and IV. The data suggest that the macrophage content of intimal area of the aorta increased with high cholesterol diet and that Rapamune did not alter the high cholesterol diet-induced increase in the macrophage content of intimal area.

Discussion

Similar gains in weight in the four groups suggest that food consumption probably was similar in all the groups, and cholesterol or Rapamune had no effect on body weight for this duration of study. A high cholesterol diet elevated the levels of serum TG, TC, LDL-C, and HDL-C. Similar changes in these parameters have been reported earlier by other investigators.^{7,17} Rapamune had no effect on serum lipids in normocholesterolemic rabbits. However, it did reduce the serum levels of TG, TC, LDL-C, and HDL-C in hypercholesterolemic rabbits. The present data on lipids are opposite to that reported earlier by various investigators. Rapamune significantly increases the plasma cholesterol levels in apo E-deficient (apo E^{-/-}) mice.¹⁸ Elloso et al¹⁹ reported a 30% increase in LDL-C and a dose-dependent increase in HDL-C. However, others have not shown similar results.^{20,21} In patients, all the data show an increase in serum lipids with Rapamune.

Rapamune in renal transplant patients increased the serum levels of TG and TC,²² TC and LDL-C,²³ and TG.²⁴ Hyperlipidemia was dose dependent in renal transplant recipients and was reversible within 1 to 2 months after discontinuation of treatment with Rapamune.²⁴ It is surprising that Rapamune in our present study did not increase the serum levels of lipids. In hyperlipidemic rabbits, Rapamune did decrease the serum levels of lipids. This is similar to the results in apo E-deficient mice reported earlier.^{20,21} It appears that the effects of Rapamune on serum lipids are very variable and may be related to animal species. It also suggests that reported antiatherogenic effect of Rapamune may not be due to alteration in serum lipids and may be due to alteration in other risk factors implicated in atherosclerosis.

The levels of serum MDA were higher in the high cholesterol diet group compared with the control group suggesting that hypercholesterolemia elevated oxidative stress. Increases in the serum levels of MDA with high cholesterol diet have also been reported earlier.^{10,17} Rapamune in the present study reduced the serum levels of MDA. Rapamune has also been reported to reduce the serum levels of MDA in rat.²⁵ It is surprising that serum protein carbonyls, another measure of oxidative stress was elevated with Rapamune in the present study. To the best of our knowledge no other data are available for serum protein carbonyls. The lowering of serum MDA with Rapamune in cholesterol treated rabbits may be due to MDA binding to protein forming protein

Fig. 2 Representative photographs of the histological changes showing the intima and media stained with hematoxylin-eosin. Magnification is shown in the figure. Group I, control; group II, 1% cholesterol diet; group III, control + Rapamune; and group IV, 1% cholesterol + Rapamune.

carbonyls which was elevated. Aortic MDA was not affected but aortic protein carbonyls were elevated with Rapamune in hypercholesterolemic rabbits in the present study. The protein carbonyl data for serum and aorta suggest that Rapamune may be atherogenic. No other data are available for aortic MDA and protein carbonyls with Rapamune.

The severity of atherosclerotic lesions in the aorta was associated with hypercholesterolemia in the present study. Similar observations have been reported by other investigators in hypercholesterolemic rabbits.^{17,26} All parameters measured (intimal atherosclerotic plaque, intimal and medial thickness, I/M ratio, and macrophage accumulation in the intima) in our study show that Rapamune does not alter the extent of high cholesterol diet-induced atherosclerosis. However, there is consensus that Rapamune reduces that aortic atherosclerotic plaque in apo E-deficient mice.^{18–21} There is one study where Rapamune has been shown to maintain neointimal hyperplasia at least up to 6 months after SES implantation in diabetic porcine.¹⁴ Neointimal formation after balloon-induced vascular injury in Yucatan minipigs is reduced by oral rapamycin.^{12,13} These differences in the data could be due to differences in animal species and strain. The data appear to be inadequate and conflicting as far as the effects of Rapamune in atherosclerosis are concerned.

There was an extensive macrophage accumulation in the intima of the aorta of rabbits on the high cholesterol diet and Rapamune did not alter the macrophage accumulation in the

intima of aorta from the rabbits on high cholesterol diet in the present study. However, a decrease in macrophage accumulation in the atherosclerotic lesions has been reported in Rapamune treated apo E-deficient mice.^{19–21} Sirolimus stent implanted in atherosclerotic arteries of cholesterol fed rabbits selectively clear the macrophage plaque.²⁷ The data are conflicting and these differences could be due to animal species differences.

In the hypercholesterolemic rabbit models of atherosclerosis, Rapamune had no effect on the extent of atherosclerosis in spite of reduction in serum lipids. All the parameters of atherosclerosis, that is, intimal plaque formation, intimal thickness, I/M ratio, macrophage accumulation, remained unaltered with Rapamune in hypercholesterolemic rabbits. Rapamune has been reported to induce hypercholesterolemia and hypertriglyceridemia^{18,19,22,23,28} and hyperglycemia.²⁹ Hypercholesterolemia is known to induce atherosclerosis.^{7,17,30} Hypertriglyceridemia have been implicated in the development of atherosclerosis.^{8,31} Hyperglycemia induces atherosclerosis through increasing the serum levels of advanced glycation end products (AGEs). The AGEs and receptor for AGEs axis is involved in the development of atherosclerosis.^{9,32} Rapamune has paradoxical proinflammatory effects on human monocytes and amyloid dendritic cells by enhancing the expression of proinflammatory cytokines (interleukin [IL]-12, IL-23, or IL-6) and blocks anti-inflammatory cytokine IL-10. Cytokines have been implicated in the development of atherosclerosis.¹⁰

Fig. 3 Histological sections of aorta from four experimental groups of rabbits stained with Movat stain. Note the black stained elastic fibers. EEL, external elastic lamina; IEL, internal elastic lamina. Magnification is shown in the figure. Group I, control; group II, 1% cholesterol diet; group III, control + Rapamune; and group IV, 1% cholesterol + Rapamune.

Fig. 4 Histological sections of aortic ring from four experimental groups of rabbits stained with macrophage stain with low magnification. Note the brown stained macrophage protein. Magnification is shown in the figure. Group I, control; group II, 1% cholesterol diet; group III, control + Rapamune; and group IV, 1% cholesterol + Rapamune.

Fig. 5 Representative photographs of part of the histological sections of aorta from the four experimental groups stained with macrophage stain with high magnification. Note brown stained macrophage. Magnification is shown in the figure. Group I, control; group II, 1% cholesterol diet; group III, control + Rapamune; and group IV, 1% cholesterol + Rapamune.

Considering hypercholesterolemia, hypertriglyceridemia, hyperglycemia, and increased proinflammatory cytokines with Rapamune, one would have expected an increase in the extent of atherosclerosis. However, Rapamune in our study did not alter the extent of atherosclerosis. This could be because of the balance between proatherogenic and anti-atherogenic effects of Rapamune.

The other factors which may favor atheroprotective effects of Rapamune include the followings. Rapamune inhibits

smooth muscle cell migration⁴ and inhibits monocyte chemotaxis.²⁰ Rapamune inhibits uptake of lipid, increases cholesterol efflux, and inhibits inflammatory cytokines.³³ Rapamune is known to overexpress endothelial nitric oxide synthase (eNOS).³⁴ Rapamune directly inhibits smooth muscle cell proliferation.³ Smooth muscle cell proliferation and migration in the intima are critical for initiation and progression of atherosclerosis.

Th1 cells promote macrophage activation and cell-mediated immune responses through production of cytokines interferon- γ but Th2 cells produce cytokines that are anti-inflammatory, promote production of IL-4 and IL-10.³⁵ Atherosclerosis is reduced with inhibition of Th1 responses.³⁶ Rapamune suppresses T cell responses.¹⁹ Rapamune increases the expression of transforming growth factor- β 1 expression,¹⁹ which has antiproliferative effects on T cells and other cells involved in the development of atherosclerosis.³⁷

In conclusion, the data suggest that hypercholesterolemic atherosclerosis is associated with an increase in oxidative stress. Rapamune reduces the serum lipid levels but increases oxidative stress in hypercholesterolemic rabbits. Rapamune did not alter the extent of atherosclerosis in spite of elevated levels of oxidative stress.

Fig. 6 Macrophage content of the intimal area of the aortic cross sections from the four experimental groups expressed as percentage of the total intimal area. Group I, control; group II, 1% cholesterol diet; group III, control + Rapamune; and group IV, 1% cholesterol + Rapamune. The results are expressed as mean $\pm$ standard deviation. * $p < 0.05$, group I versus group II, group III, and group IV. † $p < 0.05$, group II versus group III and group IV. ** $p < 0.05$, group III versus group IV.

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