Mycophenolate mofetil treatment reduces cholesterol-induced atherosclerosis in the rabbit

Freddy Romero, Bernardo Rodríguez-Iturbe *, Héctor Pons, Gustavo Parra, Yasmir Quiroz, Jaimar Rincón, Luisandra González

Department of Immunobiology, Division of Organ Transplantation, Instituto de Investigaciones Biomédicas (FUNDACITE-Zulia), Universidad del Zulia and the Renal Service, Hospital Universitario, Maracaibo, Venezuela

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Abstract

Immunosuppressive therapy has been shown to either improve or, more frequently, enhance the development of atherosclerosis. We tested the effect of mycophenolate mofetil (MMF), an inhibitor of nucleotide synthesis widely used in transplant therapy, in diet-induced atherosclerosis in the rabbit. Two groups (n=10 each) of New Zealand White (NZW) rabbits were fed a 1% cholesterol diet for 12 weeks. One group received MMF (CHOL + MMF group) by gastric gavage (30 mg/kg daily) and the other group (CHOL) received the same volume of saline by the same route. There were no differences in the serum cholesterol (mean values ≥30 mmol/l in both groups after 2 weeks) or in the triglyceride, blood sugar, total protein, and albumin serum levels and weight gain in both groups of animals. The cholesterol-fed untreated rabbits had atherosclerotic plaques covering 43.9±16.40% of their thoracic aorta and 41.9±22.59% of their abdominal aorta, while the MMF treated group had 18.5±7.17% and 17.7±9.71%, respectively (P<0.01). The cholesterol content of the aorta (mg/g) in the cholesterol-fed untreated group was 4.61±SD 1.21 in the thoracic aorta and 4.54±2.07 in the abdominal aorta, whereas the MMF treated group had 2.83±0.84 and 2.77±1.44, respectively (P<0.01). Infiltrating macrophages (RAM 11 positive cells/100 nuclei) in the intimal layer of the aorta were 58.4±SD26.16 in the CHOL group and 8.5±5.51 in the CHOL + MMF group; (P<0.001). CD18 positive cells:100 nuclei were 27.4±17.6 in the CHOL group and 5.3±3.82 in the CHOL + MMF group (P<0.01), and the intima/media ratio was 0.66±0.11 in the CHOL group and 0.30±0.09 in the MMF treated rabbits (P<0.001). MMF also reduced proliferating smooth muscle cells (HHF35 positive) infiltrating between the macrophages. These results indicate that MMF ameliorates importantly the atherogenic potential of a high cholesterol diet and this effect is associated with a reduction in macrophage and foam cell infiltration and smooth muscle cell proliferation and infiltration. Since chronic treatment with this drug is given routinely in various clinical conditions with relatively minor side effects, consideration may be given to its use as adjuvant therapy in arteriosclerotic cardiovascular disease. © 2000 Elsevier Science Ireland Ltd. All rights reserved.

Keywords: Mycophenolate mofetil in atherosclerosis; Inflammation and atherosclerosis; Macrophages in atherosclerosis

1. Introduction

The development of atherosclerosis involves an inflammatory reaction with infiltration and activation of immune competent cells [1]. However, suppression of cellular immunity has resulted usually [2–5], though not always [6], in enhancement of atherosclerosis in animals fed with a high cholesterol diet. Mycophenolate mofetil (MMF) is a drug whose active component, mycophenolic acid, inhibits inosine 5′-monophosphate dehydrogenase. Since this enzyme controls the synthesis of guanosine triphosphate, MMF suppresses de novo purine synthesis, thereby exerting a selective and reversible antiproliferative activity in macrophages and lymphocytes [7]. In addition, MMF also suppresses the expression of adhesion molecules that are critical for the migration of leukocytes from circulation to the tissues [8]. We [9] and others [10] have recently shown that MMF prevents macrophage infiltration in the remnant kidney model, and it seemed plausible that similar
effect could be achieved in the aorta of rabbits fed with a high cholesterol diet. If so, a reduction in atherosclerotic disease was a likely consequence because macrophage-derived foam cells are the most important cellular component in the atherosclerotic lesion [11–13]. Here we report that MMF ameliorates importantly the atherogenic potential of a high cholesterol diet suppressing macrophage infiltration and smooth muscle cell proliferation. Since chronic treatment with this drug has relatively minor side effects [14,15], these findings suggest therapeutic applications of this drug in hypercholesterolemic conditions with a high risk of potentially fatal cardiovascular complications in man.

2. Methods

2.1. Animals, diet and experimental design

Studies were done in 20 male New Zealand White (NZW) rabbits, which weighed 1.0–1.5 kg at the beginning of the experiment. After obtaining baseline blood samples, they were fed ad libitum a 1% Cholesterol diet for 12 weeks. This diet was prepared dissolving cholesterol (Sigma, St. Louis, MO) in 100% ethanol at a temperature of 60°C, mixing this solution with standard rabbit chow (Purina) and allowing the complete evaporation of the ethanol.

The rabbits were divided in two groups: (1) CHOL + MMF group (n = 10) received by gastric gavage 30 mg/kg of MMF (CellCept®, Roche Pharm. Co) in 0.4 ml of water and (2) CHOL group (n = 10) which received by the same route daily 0.4 ml water. Since the MMF is insoluble in water, the drug was individually prepared as a suspension by vigorous shaking immediately before administration as described in a previous paper [9]. The MMF and vehicle were given as described daily throughout the 12 experimental weeks.

Blood samples for determination of plasma cholesterol and triglycerides, serum creatinine, blood glucose levels, total protein and albumin concentration, were taken every 2 weeks. Blood chemistries were done by autoanalyzer methodology (Express Plus, CIBA, Corning Diagnostic Corp.). High density lipoprotein (HDL) cholesterol was determined after precipitation of low density lipoprotein (LDL) and very-low (VLDL) fractions with phosphotungstic acid–MgCl2 [16]. The combined VLDL and LDL fraction was calculated as the difference between the total cholesterol and the HDL cholesterol. Rabbits were weighed every 2 weeks.

A separate set of 10 rabbits of similar weight kept for 12 weeks on a standard rabbit chow not supplemented with cholesterol were sacrificed to determine normal values.

2.2. Preparation of tissues

Animals were sacrificed at the end of the 12th week. The aorta was rapidly dissected and cut from the beginning of the aortic arch to the bifurcation of the iliac vessels. Then, thoracic and abdominal segments of the aorta were separated using as a reference the diaphragm. Aortic rings of about 1 mm width were cut at the initiation of the aortic arch for histologic and immunohistologic analysis. Then, thoracic and abdominal segments of the aorta were open longitudinally and photographed for evaluation of the extension of atherosclerotic plaques. The adventitia was then carefully separated, and the aortic segments were weighted and used for determination of the total cholesterol content.

2.3. Determination of aortic cholesterol content

Lipids were isolated from the aortic segments as described by Folch et al. [17]. Briefly, tissue was homogenized in a mixture of chlorophorm–methanol 2:1 (v:v) in a final vol. 20 times de mixture volume. Homogenates were centrifuged at 2500 rpm for 15 min and the supernatant was washed in ionic 0.017% MgCl₂ solution and then centrifuged for 20 min. Lipids were extracted from the lower layer. Cholesterol was determined in the lipid extract by the method of Zlatkis et al. [18] as follows: 0.1 ml of the lipid extract was reacted with 3.0 ml acetic acid 100% and 2.0 ml ferric chloride solution. This mixture was vigorously agitated at room temperature and absorbance was measured at 560 nm (Shimatzu spectrophotometer model UV2100S, Kyoto, Japan). Calibration curves were prepared dissolving 100 mg cholesterol (Sigma, St. Louis, MO) in 100 ml glacial acetic acid 100%.

2.4. Extension of plaque formation

The extension of plaque formation was determined using the photographs of the aortas open longitudinally. The photographs were photocopied augmented in size (usually 20–50%). Then the areas corresponding to atherosclerotic plaques and the rest of the aorta were cut separately from the photocopy and weighed. No attempt was made to cut out fatty streaks separately from the normal aortic tissue. The extension of the plaques was expressed as the ratio of the weight of the areas of plaques/weight of area of the rest of the aorta × 100. Aortas from each rabbit were evaluated in triplicate. Intra and interobserver variation coefficients were 6.9 and 10.3%, respectively. Randomly selected aortic samples were also tested superimposing templates of the aortas on a dot grid and counting dots covered by atherosclerotic plaques and dots covering normally appearing aortic tissue, as done by others [19]. Agreement between these two methods was within 12%.
Aortic ring sections were fixed in formalin as well as snap frozen in tissue freezing medium (TBSTM, Triangle Biomedical Sciences, Durham, NC, USA) and kept at −70°C. Formalin-fixed tissues were stained with hematoxylin-eosin and Periodic Acid Schiff (PAS). At least three sections from the aorta from each rabbit were analyzed and in each one of them, 15–20 different fields were studied using a graduated eye piece for the assessment of the intimal/medial thickness ratio. Results represent the mean obtained in each animal.

All histologic and immunohistologic (see later) studies were done by two investigators (GP, FR) without prior knowledge of the animal group being studied.

2.5. Immunohistologic techniques and antibodies

Immunohistology was done in 4 µm thick frozen sections by the avidine biotine peroxidase methodology as described in previous communications [20]. The following monoclonal antibodies (Mab) were used: Mab anti rabbit macrophages (clone RAM11, concentration 3 µg/ml; Accurate Chemical and Scientific Corp., Westbury, NY), Mab anti CD54 (clone RR1/1, concentration 25 µg/ml; specificity = human and rabbit ICAM-1, Bender Medsystems, Vienna, Austria), Mab anti rabbit CD18 (clone L13/64, concentration 5 µg/ml; distribution in rabbit macrophages, neutrophils and platelets, Research Diagnostics Inc., Flanders, NJ), Mab anti muscle cell actin (clone HHF-35, concentration 5 µg/ml; specificity = alfa actin from skeletal, cardiac and smooth muscle cells and gamma actin from smooth muscle source, Accurate Chemical and Scientific Corp., Westbury, NY, USA) and mouse anti proliferating cell nuclear antigen (PCNA) (clone PC10, concentration 5 µg/ml Zymed Lab. Inc., San Francisco, CA). Staining with anti-CD54, anti-CD18 and anti-PCNA Mabs were standardized using rabbit lymph node frozen sections, while anti-muscle cell actin Mab was standardized in frozen sections of rabbit atherosclerotic plaques (thoracic aorta). The following negative controls were used: (a) normal rabbit aorta and (b) aorta from rabbits with atherosclerosis in which the antibodies listed above were substituted by a non-pertinent monoclonal antibody (B1: Mab anti-human B lymphocytes, Coulter Immunology, FL) at a concentration of 5 µg/ml following the procedures described previously [20].

Positive cells and nuclei were counted and results expressed as the number of positive cells/100 nuclei. Staining for CD54 and HHF35 was graded in an arbitrary scale from 1+ to 4+, depending on the intensity and number of positive cells.

Statistical analysis was done with the help of a commercial statistical package (GraphPad Instat™). Non-parametric tests were used throughout: Wilcoxon (paired samples) and Mann–Whitney (unpaired samples) were used to test differences between the baseline and the 12-week data; Kruskal–Wallis ANOVA tests were used to evaluate jointly the findings in controls and both experimental groups (MMFx treated and untreated). Serial determinations were analyzed by non-parametric Friedman’s repeated measurements test, followed by Dunn’s multiple comparisons post-tests. Correlation between cholesterol content in the aortas and extension of atheromatous plaque distribution was explored with linear regression analysis and with Spearman’s non-parametric correlation. Results are given as mean ± SD.

3. Results

The routine blood chemistries and the body weight of the rabbits at the start and at the end of the experiment are shown in Table 1. There are no differences between

<table>
<thead>
<tr>
<th>Blood chemistries</th>
<th>Controls (n = 10)</th>
<th>CHOL group (n = 10)</th>
<th>CHOL + MMF group (n = 10)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cholesterol (mmol/l)</td>
<td>1.86 ± 0.36</td>
<td>1.72 ± 0.26 (n = 9)</td>
<td>1.65 ± 0.38</td>
</tr>
<tr>
<td>HDL (mmol/l)</td>
<td>0.75 ± 0.21</td>
<td>0.67 ± 0.23 (n = 9)</td>
<td>0.70 ± 0.18</td>
</tr>
<tr>
<td>VLD + LDL (mmol/l)</td>
<td>1.08 ± 0.20</td>
<td>1.04 ± 0.25 (n = 9)</td>
<td>0.95 ± 0.26</td>
</tr>
<tr>
<td>Triglycerides (mmol/l)</td>
<td>1.58 ± 0.34</td>
<td>1.69 ± 0.33 (n = 9)</td>
<td>1.53 ± 0.46</td>
</tr>
<tr>
<td>Creatinine (µmol/l)</td>
<td>84.9 ± 15.7</td>
<td>83.1 ± 17.7</td>
<td>91.1 ± 17.6</td>
</tr>
<tr>
<td>Glucose (mmol/l)</td>
<td>6.20 ± 0.92</td>
<td>6.01 ± 1.27</td>
<td>5.76 ± 0.61</td>
</tr>
<tr>
<td>Total protein (g/l)</td>
<td>56.8 ± 4.34</td>
<td>61.8 ± 3.05</td>
<td>59.3 ± 4.00</td>
</tr>
<tr>
<td>Albumin (g/l)</td>
<td>32.2 ± 8.69</td>
<td>39.9 ± 5.96</td>
<td>35.0 ± 4.00</td>
</tr>
<tr>
<td>Body weight (kg)</td>
<td>1.39 ± 0.20</td>
<td>2.30 ± 0.15**</td>
<td>1.22 ± 0.15</td>
</tr>
</tbody>
</table>

* There are no significant differences between experimental groups (CHOL, high cholesterol diet; CHOL + MMF, high cholesterol diet + mycofenoate mofetil) and control group in baseline data. Data at 12 weeks in the CHOL and CHOL + MMF groups are not significantly different. ** P < 0.01 increments between baseline and 12 weeks in the individual groups. Data are mean ± SD.
the untreated and the MMF-treated rabbits. Control and experimental groups gained weight similarly during the 12 weeks of the experiment. As shown in Table 1, both experimental groups increased significantly ($P < 0.01$) the levels of total cholesterol, VLDL + LDL, HDL, and triglycerides after 12 weeks of cholesterol supplemented diet. However, total cholesterol increased about 16 times, while HDL and tryglicerides increased 1.5–1.6 times. There are no significant differences between the group treated with MMF and the untreated group.

Plasma cholesterol changes are shown in Fig. 1. Levels increased up to the 4th week with the high cholesterol diet and then remained at levels close to 30 mmol/l until the end of the study. There were no significant differences between the CHOL and CHOL + MMF groups.

The cholesterol content and the extension of atherosclerotic plaques in both the thoracic and abdominal aorta were significantly reduced by the treatment with MMF (Table 2). There was a significant correlation between the aortic cholesterol content and the extension of atherosclerotic plaques in the abdominal aorta ($r = 0.494$, $P = 0.031$) and in the thoracic aorta ($r = 0.768$, $P < 0.0001$). The later correlation is shown in Fig. 2 [deleted].

Histological evaluation showed that animals treated with MMF had about half the value of intima/media ratio than the untreated group (Fig. 3, right side). Infiltration of RAM11 positive cells and LFA1 positive cells in the intima was seven times and five times, respectively, more intense in the untreated CHO group than in the CHO + MMF group (Fig. 2, left side). Similarly, intense staining with smooth muscle cell specific actin was present in the untreated rabbits and substantially reduced with MMF. Macrophage infiltration in the media was also higher in the CHOL group (10.6 ± SD6.87 RAM11 positive cells/100 nuclei) than in the CHO + MMF group (0.76 ± 0.85, $P < 0.01$). Representative examples of histology are shown in Fig. 3(A–F).

Proliferating cells (PCNA positive cells/100 nuclei) were evaluated in six control rabbits, five cholesterolfed rabbits untreated with MMF and in five choleste-

### Table 2

<table>
<thead>
<tr>
<th>Cholesterol content (mg/g aorta)</th>
<th>% Occupied by plaques</th>
<th>Experiment-Thoracic</th>
<th>Abdominal</th>
<th>Thoracic</th>
<th>Abdominal</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cholesterol 2.82 ± 0.84** 2.77 ± 1.47** 18.5 ± 7.17** 17.7 ± 9.71**</td>
<td>41.9 ± 22.59</td>
<td>4.61 ± 1.21 4.54 ± 2.07</td>
<td>43.9 ± 16.4</td>
<td>4.19 ± 22.59</td>
<td></td>
</tr>
<tr>
<td>+ MMF</td>
<td></td>
<td></td>
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</table>

a Normal values for cholesterol content (mg/g of aortic tissue) in rabbits with a diet not supplemented with cholesterol ($n = 10$) are 2.18 ± 0.35 and 1.43 ± 0.15, for thoracic aorta and abdominal aorta, respectively.

** $P < 0.01$, values are mean ± SD.

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Fig. 1. Plasma cholesterol levels. Similar hypercholesterolemia was present in the cholesterol-fed animals treated with Mycophenolate mofetil (open circles, interrupted line) and the untreated animals (closed circles, continuous line). Data are mean ± SD. Values in both groups after the second week represent a significant increment ($P < 0.001$) above baseline determinations (B).

Fig. 2. Effect of MMF on the intensity of macrophage infiltration, expression of adhesion molecule CD18 in the intima and intima/media aortic ratio. Open bars, control rabbits in normal diet; Closed bars, high cholesterol diet; Hatched bars, high cholesterol diet and MMF treatment. Left side, macrophage infiltration indicated by the number of RAM 11 positive cells ($n = 9$ in the untreated group, $n = 8$ in the MMF-treated group and $n = 8$ in the control group) and expression of CD18 adhesion molecule in the aortic intima ($n = 8$ in all groups) are expressed as a ratio of positive cells/100 nuclei. Right side, the size and severity of the atherosclerotic lesions is reflected by the intima/media ratio, which was significantly reduced by MMF treatment. The values found in the untreated hypercholesterolemic rabbits ($n = 10$) ranged from 0.5 to 1.0, which correspond to relatively advanced lesions; in contrast, the intimal/media ratio in the MMF-treated rabbits ($n = 8$), ranged from 0.1 to 0.4, which represent an early fatty streak consisting of mixed macrophages and smooth muscle cells [13]. All data shown are mean ± SD. *** $P < 0.001$ vs. MMF-treated and control groups; * $P < 0.001$ vs control.
Fig. 3. Effect of MMF treatment on intimal thickness, macrophage infiltration and smooth muscle cell proliferation. Intimal thickness compared in hematoxilin & eosin staining of the intima in untreated (4A) and MMF-treated (4B) rabbit. Macrophage infiltration, evidenced by the large number of RAM 11 positive cells in untreated rabbit (4C), is reduced with MMF treatment (4D). Intense alfa actin smooth muscle cell staining (HHF-35 Mab) in the intima in the untreated rabbits (4E) is reduced by MMF treatment (4F). Bar in each panel indicates 25 μm.
terol-fed rabbits treated with MMF. They were scarce in the aorta of control rabbits (intima = 0.7 ± 0.51, media = 0.7 ± 1.21) but prominent in cholesteryl-fed rabbits (intima = 50.2 ± 8.2, media = 16.4 ± 1.67, P < 0.01 vs. control). Treatment with MMF reduced the number of PCNA positive cells (intima 28.2 ± 10.10, media = 7.0 ± 3.81; P < 0.05 and P < 0.01, respectively, versus the corresponding findings in cholesteryl-fed untreated rabbits).

Intimal expression of CD54 adhesion molecule (gradated from 0 to 4+) was higher in the untreated group (3.0 ± 0.82) and in the MMF treated group (1.93 ± 1.25) but this difference did not reach the established level of significance.

4. Discussion

The cholesteryl-fed rabbit is a model frequently used to study the development and modifications in the natural history of atherosclerosis. We administered a 1% cholesteryl diet for 12 weeks and observed increments in plasma cholesterol and, to a lesser degree, increments in plasma triglycerides, similar to those reported in the literature with similar diets [21,22]. No other biochemical abnormality was detected in our serial studies.

Since the early events in the development of atherosclerosis include a local proliferative reaction [1] with leukocyte adhesion and infiltration of lipid-containing macrophages beneath the endothelium [13,23–25], a drug that could block these events would likely retard or diminish plaque formation. For this purpose we selected MMF because in previous experiments in rats with reduced nephron mass, MMF administration given at the dose and by the route selected here was well tolerated and resulted in a substantial reduction of the renal inflammatory infiltrate [9]. In the present studies only two rabbits presented diarrhea that subsided after stopping MMF for 3 days and did not reappear after restarting the drug. The rabbits taking daily MMF and the cholesteryl-fed untreated rabbits, had comparable weight gain in the 12 weeks of the studies (Table 1).

Total cholesterol content of the aorta, is a good indirect measure of atherosclerotic severity in cholesteryl-fed rabbits and the separation of total cholesterol in the aorta into free and esterified fractions is not superior to total cholesterol alone [26]. In fact, in the present work there was a significant correlation between the aortic cholesterol content and the extension of plaque formation, particularly in the thoracic aorta. The extension of plaque formation was determined using their relative weight in magnified xerox copies of pictures of the aortas. This method combines the methods used by Hata et al. [27] and Araujo et al. [28] and offers acceptable reproducibility.

The size and severity of the atherosclerotic lesions is reflected by the intima/media ratio and this ratio was significantly reduced in the MMF-treated rabbits (Fig. 2, right side and Fig. 3A and B). The values found in the cholesteryl-fed untreated group ranged from 0.5 to 1.0, which, in studies of Rosenfeld and Ross [13], correspond to relatively advanced lesions that contain the beginning of a fibrous cap and a core of foam cells [29] with proliferating smooth muscle cells containing lipid laden macrophages [24]. In contrast, the intima/media ratio in the MMF treated rabbits ranged from 0.1 to 0.4 (Fig. 2) which represent a fatty streak consisting of mixed macrophages and smooth muscle cells [13].

It is now recognized that the macrophage-derived foam cells represent the most important cellular component in the atherosclerotic lesion [11] [25] [29–34]. Therefore, the drastic reduction in these cells in the MMF treated group is a likely explanation for the reduction in cholesterol content and plaque extension in the aorta of rabbits treated with MMF.

In man, long term administration of MMF is well tolerated in chronic conditions such as transplant immunosuppression and refractory rheumatoid arthritis [14,15]. Our studies, showing an important reduction in atherogenesis and cellular infiltration and proliferation in cholesteryl-fed rabbits, are in line with a recent preliminary report that suggested a trend towards reduction in plaque formation by the subcutaneous administration of mycophenolic acid [35]. We suggest that MMF may be of value in clinical conditions characterized by a very high plasma cholesterol level and a high associated risk of ischemic heart disease, such as familial hypercholesterolemia. Indeed, drugs with more significant side effects and several invasive surgical procedures with a high rate of complications are presently used in the treatment of this condition.

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References
