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Cholesterol diet and effect of long-term withdrawal on plaque development and composition in the thoracic aorta of New Zealand White rabbits

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ABSTRACT

Aims: Experimental study on plaque progression, regression and composition in atherosclerotic thoracic aorta of hypercholesterolemic rabbits after long-term withdrawal of cholesterol-enriched diet (CED).
Methods: Rabbits were fed 2% cholesterol for 6 weeks followed by withdrawal periods for 15, 23, 34, 68, or 78 weeks. Cholesterol, triglyceride, and phospholipids levels in blood and cholesterol concentrations in aorta were quantified. Plaque size and cellularity, phenotype of macrophages and smooth muscle cells were (immuno)histomorphometrically analyzed in segments of the thoracic aorta.
Results: After 6 weeks of CED, blood cholesterol levels were about 80-fold higher, whereas atherosclerosis and cholesterol content in the thoracic aorta were only minimally increased. However, the latter significantly increased within 15 weeks after cholesterol withdrawal, while serum cholesterol level was still 10-fold increased. Thereafter plaque area and cholesterol content remained almost unchanged until the end of the study despite a long-term normalization of serum cholesterol level after withdrawal of CED. Directly after 6 weeks of CED the densities of macrophages and apoptotic cells within plaques were highest, decreasing after cholesterol withdrawal, whereas, *vice versa* the density of smooth muscle cells (SMCs) significantly increased.
Conclusion: We suggest that atherosclerotic plaques respond to long-term withdrawal of CED by decrease in number and phenotype of macrophages and increase of SMCs without regression of the lesion size. The cellular changes are suggested to considerably contribute to higher plaque stability.

1. Introduction

High blood cholesterol level is suggested as independent cardiovascular risk factor and its lowering is a major goal of an anti-atherosclerotic therapy. However, recent studies reveal that atherosclerosis reduction can also be pharmaceutically achieved without affecting lipid levels, suggesting that cellular effects or other factors like inhibition of oxidative stress in the atherosclerotic vessel wall may prevent progression [1–3]. However, cellular composition might not only influence plaque growth, but also its stability. Findings in experimental and human atherosclerotic lesions suggest that increased macrophage activities mostly associated with inflammatory processes, an extracellular lipid accumulation, or decreased smooth muscle cell (SMC) density might be the pathogenic basis for plaque instability [4,5].

Effects of increased blood cholesterol levels on atherosclerosis development were often investigated by the use of animal models e.g. using New Zealand White (NZW) rabbits, which were fed a CED for a certain period of time [2,3,6,7]. The purpose of our study was to investigate effects of a short-term feeding of high (2%) CED [8] and thereafter of its long-term withdrawal for up to 78 weeks on cholesterol, triglyceride, and phospholipids levels in blood, and induction, progression and regression of atherosclerotic plaques lesions. Moreover, plaque composition was studied by measuring cholesterol content, cellularity, macrophage phenotype, SMCs, and apoptosis, which have all been suggested to influence plaque stability. As an indicator for oxidative stress manganese superoxide dismutase (MnSOD) was investigated in atherosclerotic lesions [6,10].

Abbreviations: CED, cholesterol-enriched diet; ir, immunoreactivity; LDL, low-density lipoproteins; MnSOD, manganese-superoxide dismutase; NZW, New Zealand White rabbits; SC, standard chow; TUNEL, TdT-mediated dUTP nick end labeling; ox-LDL, oxidized low-density lipoproteins; SMC, smooth muscle cell.

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Table 1
Treatment and blood lipid concentrations of the animals of groups I–VII.

Group	*n*	CED (6 weeks)	SC	Chol. [mg/dl]	Trigl. [mg/dl]	Phos. [mg/dl]	C/P
I	10	–	–	35.1 ± 2.5	54.9 ± 8.3	70.9 ± 3.8	0.50 ± 0.03
II	15	×	–	2878.6 ± 221.7***	146.4 ± 29.9***	814.5 ± 75.5***	3.60 ± 0.12***
III	10	×	15 weeks	355.9 ± 95.0$^{**,\#\#\#}$	48.7 ± 12.7$^{\#\#\#}$	181.2 ± 37.1$^{**,\#\#\#}$	2.80 ± 0.17$^{***,\#\#\#}$
IV	10	×	23 weeks	100.5 ± 42.3$^{*,\#\#\#}$	46.3 ± 9.3$^{\#\#\#}$	77.5 ± 13.9$^{\#\#\#}$	1.73 ± 0.21$^{***,\#\#\#}$
V	10	×	34 weeks	70.7 ± 50.4$^{\#\#\#}$	45.2 ± 5.3$^{\#\#\#}$	66.7 ± 22.4$^{\#\#\#}$	1.03 ± 0.18$^{***,\#\#\#}$
VI	6	×	68 weeks	23.3 ± 2.1$^{\#\#\#}$	41 ± 2.8$^{\#\#\#}$	43.2 ± 3.2$^{***,\#\#\#}$	0.60 ± 0.16$^{\#\#\#}$
VII	5	×	78 weeks	13.8 ± 3.6$^{***,\#\#\#}$	38.4 ± 7.4$^{\#\#}$	42.2 ± 4.7$^{***,\#\#\#}$	0.31 ± 0.08$^{**,\#\#\#}$

CED (6 weeks), cholesterol-enriched diet, 6 weeks; SC, standard chow; Chol., serum cholesterol; Trigl., serum-triglycerides; Phos., serum-phospholipids; C/P, cholesterol/phospholipids ratio; mean ± SEM.
* $P < 0.05$ vs. group I.
** $P < 0.01$ vs. group I.
*** $P < 0.001$ vs. group I.
$^{\#\#}$ $P < 0.01$ vs. group II.
$^{\#\#\#}$ $P < 0.001$ vs. group II.

2. Methods

2.1. Animal procedures

NZW rabbits (16 weeks old) weighing about 3–5 kg were achieved from Charles River Farm (Sulzfeld, Germany), individually housed under the same conditions, with dark–light cycles of 12 h and divided into six groups of 5–10 animals (Table 1).

Animals of the control group I ($n = 10$) received standard chow (SC) for 6 weeks and were immediately sacrificed thereafter. From the animals, which were supplemented with 2% CED for 6 weeks, group II ($n = 15$) was sacrificed immediately after the cholesterol supplementation period, and groups III–VII after cholesterol withdrawal for 15 (group III; $n = 10$), 23 (group IV; $n = 10$), 34 (group V; $n = 10$), 68 (group VI; $n = 6$), or 78 weeks [1.5 years] (group VII; $n = 5$).

This investigation conforms to the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996). All animal studies were performed in compliance with the German laws relating to the conduct of animal experimentation.

2.2. Determination of blood lipid levels

Blood was drawn from the ear vein using EDTA tubes to determine cholesterol, triglyceride and phospholipids levels using commercially available test kits (Merck, Darmstadt; Biomed, Munich; Wako, Neuss, Germany).

2.3. Aortic tissue of NZW rabbits

The thoracic aorta was dissected free of adventitial tissue. In detail, the thoracic aorta was proximo-distally divided into 10 equally sized segments (1–10). Each of these segments was divided in three subsegments: the proximal one was embedded in methacrylate (morphometry), the middle one was shock-frozen in liquid nitrogen cooled isopentane and stored at −70 °C until used (immunohistology and morphometry), and the distal one was snap-frozen in liquid nitrogen and stored at −20 °C (biochemical analysis).

2.4. Determination of cholesterol content in segments of the thoracic aorta

The distal part of the third aortic subsegments of groups I–VI was minced, extracted by the Folch procedure [9], and total cholesterol was determined using available test kits (Roche, Mannheim, Germany).

2.5. (Immuno)histology

Methacrylate cross sections (4 μm) of the proximal subsegments of the aortic segments were stained with hematoxylin–eosin and plaque size as well as lumen stenosis were measured by computer-assisted-morphometry [6].

Immunohistology of the distal subsegments of the aortic segments was routinely performed according to procedures as previously described [6,10,11]. Immunoreactions were achieved with monoclonal antibodies (mAbs) directed against α-actin (DAKO, Glostrup, Denmark), MnSOD, or Mac-1 (Roche, Mannheim, Germany) overnight at RT. After washing in PBS the sections were incubated with biotinylated anti-mouse or anti-rat Ig and with streptavidin-peroxidase (all from Amersham, Braunschweig, Germany). Staining reaction was performed by adding a diaminobenzidine solution (Pierce, Rockford, IL, USA). Appropriate isotype-matched mAbs were used as control. Another control was carried out by the omission of the first antibody, which then abolished the immunohistochemical reaction completely.

From histological and immunohistological sections, digitalized images were obtained using an Axioplan2 imaging microscope (Carl Zeiss GmbH, Jena, Germany) and the digital high resolution imaging system AxioCam/AxioVision (Carl Zeiss GmbH, Jena, Germany).

2.6. Detection of DNA fragmentation (TUNEL technique)

DNA fragmentation was studied on 4% paraformaldehyde-fixed cryo cross sections by the TUNEL (TdT-mediated dUTP nick end labeling) technique, using the ApopTag kit (Oncor, Heidelberg, Germany) as described previously [6,7].

2.7. Morphometry

Computer-assisted morphometry was performed using a standard Olympus BH2 microscope, microscopic images were recorded by a video-camera (Olympus HCC-3600 P high gain), and digitized by a personal computer equipped with an image analysis system (VIBAM 0.0 – VFG 1) developed in our group [12]. Plaque area and the number of plaque cells were determined in cross sections of the proximal subsegments of the aortic segments. The plaque area was measured by tracing the internal elastic lamina and the luminal circumference [12]. The number of plaque cells was analysed by counting all cells of a cross section.

2.8. Statistical analysis

The results were calculated as mean + SEM. All statistical procedures were performed using the SIMSTAT program (Provalis Research, Montreal, Canada). Statistical significance was deter-