CHAPTER 8

Stage-specific remodelling of atherosclerotic lesions upon cholesterol lowering in LDL receptor knockout mice

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Abstract

Lipid lowering reduces cardiovascular morbidity and mortality. However, the dynamic remodeling of established atherosclerotic lesions upon lipid lowering is poorly understood. Early and advanced lesions in the aortic root were induced by feeding LDL receptor knockout mice a high fat/high cholesterol Western-type diet for 5 and 9 weeks, respectively. Lipid lowering was achieved by switching mice to chow diet. In the first week after the diet switch, plasma total cholesterol (TC) levels dropped 70%, but both early and advanced lesions increased in size. Early lesions grew due to an increase in smooth muscle cells while advanced lesions showed an enlargement of the absolute macrophage area. From 1 to 3 weeks, plasma TC levels were completely normalized. This did not influence the size of early lesions. However, advanced lesions became smaller due to a reduction of the absolute macrophage area. From 3 to 6 weeks, both early and advanced lesions progressed further as a result of expansion of the absolute collagen and necrotic core area. In contrast, early lesions became proinflammatory as evidenced by the increased infiltration of neutrophils, increased oxidative stress, probably caused by the activation of mast cells in the adventitia.

In conclusion, the severity of the atherosclerotic lesion affects its dynamic response to lipid lowering, indicating the importance of establishing stage-specific therapeutic protocols for the treatment of atherosclerosis.

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Introduction

Atherosclerosis, the leading cause of morbidity and mortality in the Westernized society, is a multifactorial complex disease with numerous etiologies that work synergistically to promote lesion development [1]. Among the many cardiovascular risk factors, hypercholesterolemia, especially due to elevated levels of very-low-density lipoprotein cholesterol (VLDL-C) and LDL-C, is sufficient to initiate and promote atherogenesis even in the absence of other known risk factors [2]. Clinical trials have shown that the extent of LDL-C lowering is negatively associated with the rate of atherosclerosis progression [3-5]. However, after normalization of hypercholesterolemia by lipid lowering therapy, a considerable proportion of patients still showed continued progression of atherosclerosis and the occurrence of clinical events, such as myocardial infarction [6-7]. In the ASTEROID trial, after intensive LDL-C lowering by rosuvastatin, approximately 35% of the patients still showed atherosclerotic lesion progression in the coronary artery [3,8]. Also the recent METEOR study showed that intensive LDL-C lowering by rosuvastatin only reduced rather than stopped the progression of carotid atherosclerosis in asymptomatic subjects with low risk of cardiovascular disease [9]. Thus, there is a clear need for a better insight into the mechanisms underlying the effects of plasma lipid lowering on atherogenesis.

The progression of an atherosclerotic lesion is a dynamic process, involving the influx and efflux of lipids, cell migration and emigration, cell proliferation and death, and matrix synthesis and degradation [10]. Identification of lesion dynamics is expected to have important implications for the treatment of atherosclerosis, as it will allow establishment of the optimal timing for modulating specific cellular or acellular components of the lesion to induce lesion stabilization or regression. In the current study, we for the first time addressed the effects of plasma lipid lowering on the dynamic remodeling of atherosclerotic lesions with different degrees of severity in LDL receptor knockout (LDLr KO) mice by applying a dietary switch from a high fat/high cholesterol Western-type diet (WTD) to regular chow diet. Evidence is provided that the complexity of the initial lesion determines its dynamic response to lipid lowering.

Methods

Mice

LDL receptor knockout (LDLr KO) mice, obtained from the Jackson Laboratory (Bar Harbor, USA) were bred at the Gorlaeus Laboratories and maintained on sterilized regular chow, containing 4.3% (w/w) fat and no added cholesterol (RM3; Special Diet Service, Witham, UK). Mice at the age of 12 weeks received a Western-type diet containing 15% (w/w) total fat and 0.25% (w/w) cholesterol (Diet W, Special Diet Services, Whitham, UK) for 5 weeks (n = 32) and 9 weeks (n = 32) to induce the development of early and advanced atherosclerotic lesions, respectively. Subsequently, 8 animals were euthanized (control group). The remaining animals were divided into 3 groups (n=8/group) and fed a regular chow for 1 (W1 group), 3 (W3 group), and 6 (W6 group) weeks, respectively. Animal experiments were performed at the Gorlaeus Laboratories of the Leiden/Amsterdam Center for Drug Research in accordance with the National Laws. All experimental protocols were approved by the Ethics Committee for Animal Experiments of Leiden University.

Lipids Analysis

After an overnight fasting-period, blood was collected by retro-orbital puncture under anesthesia. Hepatic lipids were extracted according to the method of Bligh & Dyer [11] and dissolved in 2% Triton X-100. Triglycerides (TG) and Phospholipids (PL) in serum and liver were determined using a standard enzymatic colorimetric assay (TG: Roche Diagnostics, Mannheim, Germany; PL: Spinreact, Girona, Spain). The concentrations of cholesterol in serum and liver were determined by incubation with 0.025
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U/mL cholesterol oxidase (Sigma) and 0.065 U/mL peroxidase and 15 μg/mL cholesteryl esterase (Roche Diagnostics, Mannheim, Germany) in reaction buffer (1.0 KPi buffer, pH=7.7 containing 0.01 M phenol, 1 mM 4-aminooantipyrine, 1% polyoxyethylene-9-lauryl ether, and 7.5% methanol). Absorbance was read at 490 nm. The hepatic lipids levels were normalized to their protein concentrations determined using the BCA™ protein assay (Pierce Biotechnology, Rockford, USA). The distribution of cholesterol over the different lipoproteins in serum was determined by fractionation of 30 μL of serum of individual mice using a Superpose 6 column (3.2 x 300 mm, Smart system; Pharmacia, Uppsala, Sweden).

Circulating leukocyte analysis

Upon sacrifice, blood was collected by retro-orbital puncture under anesthesia. Total white blood cells, neutrophil, lymphocyte, and monocyte counts in the blood were analyzed using an automated Sysmex XT-2000iv Veterinary Hematology analyzer (Sysmex Corporation, Kobe, Japan).

Histological Analysis of the Aortic Root

On sacrifice the arterial tree was perfused in situ with phosphate buffer solution (PBS) and the heart was excised and stored in 3.7% neutral-buffered formalin (Formal-fixx; Shandon Scientific Ltd., UK) until use. 10-μm sections were taken once the aortic root was identified by the appearance of aortic valve leaflets. The aortic arches were frozen and longitudinal 8-μm cryosections were taken. Atherosclerotic lesion development was quantified in the aortic root and aortic arch from oil red O/hematoxylin-stained cryostat sections using the Leica image analysis system, consisting of a Leica DMRE microscope coupled to a video camera and Leica Qwin Imaging software (Leica Ltd). Mean lesion area (in μm²) was calculated from 10 oil red O/hematoxylin-stained sections in the aortic root and 15 of the sections with maximal lesion area in the aortic arch. Sections were immunolabeled against MOMA-2 (monoclonal rat IgG2b, dilution 1:50, Research diagnostics), α-smooth muscle actin (monoclonal mouse IgG2a, dilution 1:500, Sigma), CD3 (polyclonal Rabbit IgG, dilution 1:150, Neomarkers), and Ly6G (monoclonal rat IgG2b, dilution 1:100, eBiosciences) for detection of monocytes/macrophages, smooth muscle cells, T lymphocytes, and neutrophils, respectively. Collagen content of the lesions was visualized with aniline blue by using Masson’s Trichrome acceustain according to the manufacturer’s instructions (Sigma). TUNEL staining of lesions was performed to visualize apoptotic cells using the in Situ Cell Death Detection kit (Roche Diagnostics). Mast cells were stained with naphthol AS-D chloroacetate esterase (Sigma). Histochemical stainings were subsequently quantified in 5 consecutive sections by computer-aided morphometric analysis using the Leica image analysis system. Furthermore, quantifications of necrotic core area were performed on oil red O/hematoxylin and Masson’s Trichrome stained sections. Oxidative stress was quantified by using α-nitrotyrosine (IgG2b, dilution 1:100, Abcam) and α-8-hydroxy-2’-deoxyguanosine (8-OHdG) (IgG2a, dilution 1:100, QED Bioscience) monoclonal mouse antibodies. All analyses were performed blinded.

Flow cytometry

White blood cell suspensions from whole blood were prepared by lysis of red blood cells. Cell surface immunolabelling of monocytes and neutrophils was performed according to the manufacturer’s instructions (eBioscience & BD Biosciences). Briefly, fluorochrome-conjugated monoclonal antibodies to CD11b (Biosciences) and to Ly6G and Ly6C (BD Biosciences) were incubated with the white blood cell suspensions for 30 min at 4°C in labeling buffer (1% mouse serum in PBS). Flow cytometric analysis was performed with FacsCalibur and then analyzed with CellQuest software (Becton Dickinson, San Jose), correcting for nonspecific staining with isotype antibody controls.

Statistical Analysis

Statistical analysis was performed using ANOVA and the Student-Newman-Keuls post-test (GraphPad InStat and Prism software). A Pearson r test was used to perform correlation analysis. A level of p<0.05 was considered significant.
Results

Dietary lipid lowering led to decreased lipid levels in plasma and liver

LDLr KO mice were fed WTD for 5 weeks to induce atherosclerotic lesion formation. Thereafter the diet was switched to regular chow to lower plasma cholesterol levels. As shown in Table 1, once the diet was switched to chow, plasma free cholesterol and total cholesterol levels dropped approximately 60% from 357±36 mg/dL to 156±2 mg/dL (p<0.001) and 70% from 1347±78 mg/dL to 438±13 mg/dL (p<0.001) at 1 week and decreased further to 84±3 and 222±6 mg/dL, respectively, at 6 weeks after the diet switch. The reduced plasma total cholesterol levels were mainly due to decreased VLDL/LDL-C levels (supplementary Figure S1). Moreover, an around 45% reduction in plasma triglycerides (p<0.001) and phospholipids (p<0.001) was observed at 1 week after the diet switch, which remained low during the remaining period of the experiment (Table 1). The reduction of plasma lipids thus mainly occurred in the first week after the dietary lipid withdrawal. In contrast, livers showed delayed cholesterol lowering upon switch of WTD to chow with the major effects seen at 3 weeks. After 3 weeks on regular chow, hepatic free and esterified cholesterol levels decreased approximately 35% (p<0.001) and 75% (p<0.001), respectively (Table 1). Dietary lipid lowering did not affect the hepatic triglyceride and phospholipid content (Table 1). Of note, dietary lipid lowering did normalize both plasma and hepatic lipid levels at 6 weeks after the diet switch to similar levels as in LDLr KO mice of the same age without WTD challenge (Table 1).

Table 1: Effect of dietary cholesterol lowering on plasma and hepatic lipids

<table>
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<tr>
<th>Groups</th>
<th>C</th>
<th>W1</th>
<th>W3</th>
<th>W6</th>
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<td>156±2***</td>
<td>96±4***</td>
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<td>1347±78</td>
<td>438±13***</td>
<td>232±7***</td>
<td>222±6***</td>
<td>237±7</td>
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<td>104±14***</td>
<td>120±10***</td>
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<td>447±23***</td>
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<td>31±4*</td>
<td>11±1***</td>
<td>9±1***</td>
<td>8±1</td>
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<td>65±3</td>
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LDLr KO mice were fed WTD for 5 weeks. Thereafter, the animals were switched to regular chow and euthanized at 0 (group C), 1 (group W1), 3 (group W3) and 6 (group W6) weeks after the diet switch. LDLr KO control mice were kept on chow diet all the time during the experiment. Plasma and hepatic lipids were measured. FC, free cholesterol; TC, total cholesterol; CE, cholesteryl ester; TG, triglycerides; PL, phospholipids. Data represent mean ± SEM of 8 mice. Statistically significant difference ***P<0.001 and *P<0.05 versus group C; †P<0.01 and ‡P<0.05 versus group W1.
Decreased circulating neutrophils and monocytes upon dietary lipid lowering

Next, we examined the effect of dietary lipid lowering on circulating leukocytes. The amount of total white blood cells was 4.0±0.7 x10^6/mL at baseline and had a tendency to decrease to 3.1±0.5 x10^6/mL at 3 weeks and remained low (3.3±0.4 x10^6/mL) until 6 weeks after the WTD withdrawal. Dietary lipid lowering did not affect the amount of lymphocytes in the circulation (~2.5x10^6/mL). As shown in Figure 1A and B, the tendency to a reduction in white blood cells by dietary lipid lowering can be attributed to reduced levels of circulating monocytes and neutrophils. Neutrophils in the circulation were reduced about 2-fold (p<0.05) after 1 week on chow, while monocytes did not change at this time point. In contrast, at 3 weeks after the dietary lipid withdrawal circulating monocytes were reduced ~3-fold (p<0.05).

Two specific monocyte subsets that vary in their capacity to infiltrate into atherosclerotic lesions are characterized by different expression levels of Ly6C. Ly6C^{high} monocytes preferentially accumulate in the growing atheroma in hyperlipidemic mice, while Ly6C^{low} monocytes do not [12]. Therefore, Ly6C^{high} and Ly6C^{low} monocyte subsets were further analyzed. Both Ly6C^{high} (3.1-fold, p<0.01) and Ly6C^{low} (3.3-fold, p<0.05) monocytes were reduced after 3 weeks on chow (Figure 1C and 1D). Moreover, the percentage of Ly6C^{high} monocytes decreased from 60±2% at baseline to 47±2% (1.3-fold, P<0.001) at 6 weeks after the diet switch.

Dynamic remodeling of established early atherosclerotic lesions upon dietary lipid lowering

Feeding LDLr KO mice the WTD for 5 weeks induced the development of early atherosclerotic lesions in the aortic root, characterized by fatty streaks and accumulation of macrophage foam cells (Figure 2C and 3A). The dynamic remodeling of these established early lesions following dietary lipid lowering was investigated by analyzing the effects on lesion size and lesion compositions at 0 (group C), 1 (group W1), 3 (group W3) and 6 (group W6) weeks after the diet switch to regular chow.
Figure 2. Dynamic remodeling of established early lesions upon dietary lipid lowering. Feeding LDLr KO mice WTD for 5 weeks induced early atherosclerotic lesion formation in the aortic root. Atherosclerotic lesion development was analyzed at the aortic root at 0 (C), 1 (W1), 3 (W3), and 6 (W6) weeks after the switch of the mice to regular chow. (A) Photomicrographs showing a scatter dot plot of atherosclerotic lesion quantification and representative oil-red-O stained sections (original magnification 10x2.5). Each symbol represents the mean lesion area in a single mouse. The horizontal bar indicates the mean value for the group. (B) Bar graphs showing the dynamic changes in macrophages, T cells, neutrophils, smooth muscle cells, collagen content and necrotic core area of early lesions after the diet switch. Statistically significant difference *P<0.05, **P<0.01, and ***P<0.001 versus group C; ###P<0.001 versus group W1; $P<0.05 and $$$P<0.001 versus group W3. (C) Pie graph showing the relative abundance of macrophage (red), smooth muscle cell (yellow), collagen (blue), necrotic core (black) and neutrophils (green) in the early lesions during the remodeling upon dietary lipid lowering. The relative percentage of each lesion component was normalized to sum to 100%.
In the initial phase (the first week after the dietary lipid withdrawal), the lesions still progressed from \(157 \pm 24 \times 10^3 \mu m^2\) to \(235 \pm 18 \times 10^3 \mu m^2\), despite the 70% decrease in plasma total cholesterol levels to 438±13 mg/dL (Figure 2A). Quantification of the lesion composition indicated a 3.7-fold increase of smooth muscle cells (p<0.05) but no significant change in macrophages, T cells or neutrophils or collagen content of lesions (Figure 2B). The percentage of apoptotic cells in the lesions was only 1.3±0.3% (Supplementary Figure S2). Morphological analysis of the lesions also revealed the appearance of a necrotic core at 1 week after the diet switch. However, the mean size of the necrotic core area was only \(2.4 \pm 0.9 \times 10^3 \mu m^2\) (Figure 2B). As shown in Figure 2C, after 1 week chow diet feeding, the early lesions were still mainly composed of macrophage foam cells (Figure 2C).

In the second phase (from 1 to 3 weeks on chow), the plasma total cholesterol levels decreased further to around 232±7 mg/dL. Interestingly, no change in the lesion size was detected as compared to 1 week chow feeding (W3: 248±18 \times 10^3 \mu m^2 vs W1: 235±18 \times 10^3 \mu m^2) (Figure 2A). Of note, dietary lipid lowering for 3 weeks did lead to a reduction in macrophages (2.1-fold, p=0.001 vs W1; 1.6-fold, p<0.05 vs C) (Figure 2B and 3B), while the average size of macrophage foam cells remained unchanged (data not shown). The smooth muscle cells (7.4-fold, p<0.05) (Figure 3C and 3D) and collagen content (7.0-fold, p<0.05) increased further as compared to baseline (Figure 2B), which formed the subluminal fibrous cap (Figure 3D). Also the percentage of apoptotic cells increased ~2.3-fold (p<0.05 vs C and W1) to 2.9±0.4% (Supplementary Figure S2). Although the buildup of necrotic material inside the lesions was augmented (5.0±2.8 \times 10^3 \mu m^2\), the collagen to necrotic core ratio was increased 3.3-fold to 16.2±5.8, indicating that dietary lipid lowering induced lesion stabilization. Moreover, T cells (Figure 3E and 3F) and neutrophils of the lesions were not significantly changed (Figure 2B and 2C). In line, the lesion contains more collagen and smooth muscle cells, and fewer macrophage foam cells after dietary lipid lowering for 3 weeks (Figure 2C).

In the third phase (from 3 to 6 weeks on chow), the size of the lesions reached a value of \(323\pm36 \times 10^3 \mu m^2\) (p<0.01 vs C) (Figure 2A), although the plasma total cholesterol levels remained low (222±6 mg/dL). The lesional macrophage area of mice kept on chow for 6 weeks increased 1.5-fold (p<0.05) as compared to the lesions of mice fed chow for 3 weeks and became similar to baseline levels (Figure 2B). The amount of smooth muscle cells in the lesions remained relatively high, 3.9-fold (p<0.05) higher as compared to baseline. Moreover, the collagen content of the lesions was further increased (2.1-fold vs W3, 14.5-fold vs C, p<0.001) (Figure 2B and 3H), indicating that the rate of collagen synthesis was still higher than that of its degradation. Importantly, in the 3 additional weeks chow diet feeding, also a dramatic enlargement of necrotic core (4.4-fold, p<0.001 vs W3) was observed (Figure 2B and 2C). The ratio of collagen to necrotic core was thus reduced 5.5-fold to 2.9±0.4 (p<0.05 vs W3). A further increase in the percentage of apoptotic cells was also found (4.7±0.6%, 1.6-fold vs W3, P<0.05; 3.7-fold vs C and W1, p<0.001) (Supplementary Figure S2). In addition, the neutrophil content of the lesions was increased 2.8-fold (p<0.001 vs W3) (Figure 2B and 3J). The enhanced infiltration of neutrophils and a decreased ratio of collagen to necrotic core suggested a proinflammatory phenotype of the lesions. Strikingly, the T cell content still remained the same as baseline (Figure 2B).

After 6 week chow diet feeding, as shown in Figure 2C, the early lesions mainly consisted of macrophages, collagen and necrotic core. Interestingly, pearson correlation analyses demonstrated that after normalization of hypercholesterolemia, the size of early
lesions was highly correlated with the macrophage ($r=0.67$, $p=0.0048$, $n=16$), collagen ($r=0.85$, $p<0.0001$, $n=16$), and necrotic core ($r=0.66$, $p=0.0055$, $n=16$) area.

Dynamic remodeling of established advanced atherosclerotic lesions upon dietary lipid lowering

We next examined whether the dynamic remodeling of established advanced atherosclerotic lesions differs from the response of early lesions upon dietary lipid lowering. Advanced lesions in the aortic roots were induced by feeding LDLr KO mice WTD for 9 weeks. The lesion size and composition were again examined at 0 (group C), 1 (group W1), 3 (group W3) and 6 (group W6) weeks after the diet switch to regular chow. When the mice were fed WTD for 9 weeks prior to the switch to chow diet, dietary lipid lowering led to a similar reduction in plasma and hepatic lipid levels as compared to 5 weeks WTD feeding prior to the diet switch (data not shown). As compared to early lesions, advanced lesions displayed a markedly lower macrophage content, increased smooth muscle cell content and collagen deposition (Figure 2C and 4C), while also a necrotic core and a fibrous cap can be distinguished. In addition, advanced lesions contained 2-fold more T cells ($p=0.0013$) and 49-fold more neutrophils ($p=0.0011$), respectively as compared to early lesions. The percentage of apoptotic cells in advanced lesions was also largely increased (8.5±0.8%, 6.7-fold, $p<0.001$) (Supplementary Figure S2). Interestingly, the neutrophil content of advanced lesions was about 5-fold ($p=0.0003$) higher as compared to the level in early lesions after 6 weeks WTD withdrawal (Figure 2B and 4B).
Figure 4. Dynamic remodeling of established advanced lesions upon dietary lipid lowering. Feeding LDLr KO mice WTD for 9 weeks induced advanced atherosclerotic lesion formation in the aortic root. Atherosclerotic lesion development was analyzed at the aortic root at 0 (C), 1 (W1), 3 (W3), and 6 (W6) weeks after the switch of the mice to regular chow. (A) Photomicrographs showing a scatter dot plot of atherosclerotic lesion quantification and representative oil-red-O stained sections (original magnification 10x2.5). Each symbol represents the mean lesion area in a single mouse. The horizontal bar indicates the mean value for the group. (B) Bar graphs showing the dynamic changes in macrophages, T cells, neutrophils, smooth muscle cells, collagen content and necrotic core area of advanced lesions after the diet switch. Statistically significant difference *P<0.05, **P<0.01, and ***P<0.001 versus group C; #P<0.05, ##P<0.01, and ###P<0.001 versus group W1; $P<0.05 and $$$P<0.001 versus group W3. (C) Pie graph showing the relative abundance of macrophage (red), smooth muscle cell (yellow), collagen (blue), necrotic core (black) and neutrophils (green) in the advanced lesions during the remodeling upon dietary lipid lowering. The relative percentage of each lesion component was normalized to sum to 100%.
In the initial phase upon WTD withdrawal, advanced lesions, increased 1.4-fold in size \((778\pm68 \times 10^3 \mu m^2 \text{ vs } 557\pm54 \times 10^3 \mu m^2, p<0.05)\) (Figure 4A). In contrast to early lesions, this was associated with a 1.7-fold \((p<0.001)\) increase of macrophage content in advanced lesions (Figure 4B). No significant change was observed in the other lesion components. Notably, in the second phase from 1 to 3 weeks on chow, a significant 1.3-fold reduction in the size of advanced lesions was observed \((606\pm50 \times 10^3 \mu m^2, p<0.05 \text{ vs W1})\) (Figure 3A), which was primarily the consequence of a 2-fold reduction in the macrophage content of the lesions (Figure 4B and 5B). In addition, T cells \((1.5\text{-fold}, p<0.05 \text{ vs W1})\) and neutrophils \((1.9\text{-fold}, p<0.05 \text{ vs W1})\) were decreased to baseline.

Increased fibrous cap area in the subluminal Moma-2 negative region (Figure 5B) was correlated with increased collagen accumulation \((2.2\text{-fold}, p<0.01 \text{ as compared to baseline})\), but smooth muscle cells and the necrotic core remained the same (Figure 4B and 4C). The collagen to necrotic core ratio was thus increased 1.8-fold to \(3.7\pm0.6 (p<0.05, \text{ vs C and W1})\). The percentage of apoptotic cells remained unchanged \((8.6\pm0.6\%)\) (Supplementary Figure S2). All these changes indicated that advanced lesions became stabilized after dietary lipid lowering for 3 weeks. In line, collagen became the major component of advanced lesions after 3 weeks chow diet feeding (Figure 4C).

In the third phase, advanced lesions, like the early lesions, progressed to \(838\pm56 \times 10^3 \mu m^2 \text{ (1.4-fold, p<0.05 vs W3)}\) (Figure 4A). This was primarily the consequence of an increase \((2.0\text{-fold, } p<0.001)\) in collagen content of advanced lesions and to a lesser extent of an enhanced buildup of necrotic core material \((1.7\text{-fold, } p<0.001)\) (Figure 4B and 5D). The macrophage area of advanced lesions did not change while smooth muscle cells

![Figure 5. Photomicrographs showing morphometric changes of advanced lesions in response to dietary lipid lowering. Sections of the aortic roots were stained with antibodies against Moma-2, α-actin, Ly6G, and CD3 to visualize macrophages (A, B, 50x), smooth muscle cells (SMCs) (E, F, 50x), neutrophils (G, H, 100x), and T cells (I, J, 100x), respectively. Morphological staining of atherosclerotic lesions in the aortic root with Masson’s Trichrome Accustain, which stains cytoplasm and muscle fiber red and collagen blue (C, D, 50x).]
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(Figure 5E and 5F) decreased 1.9-fold (p<0.05 vs W3; 2.4-fold, p<0.05 vs C) after the 3 additional weeks chow diet feeding (Figure 4B). The ratio of collagen to necrotic core remained stably high (4.0±0.4, p<0.01 vs C). The percentage of apoptotic cells in the lesions was even slightly decreased to 7.1±0.6% (p<0.05 vs W1) (Supplementary Figure S2). Moreover, neutrophils of advanced lesions (Figure 5G and 5H) were dramatically reduced (3.7-fold, p<0.05 vs W3, 5.4-fold, p<0.05 vs C) at 6 weeks (Figure 4B). T cells of advanced lesions (Figure 5I and 5J) decreased further (1.9-fold, p<0.01 vs C) to the similar levels of early lesions (Figure 4B). In line, advanced lesions became the collagen-rich lesions after dietary lipid lowering for 6 weeks (Figure 4C). Interestingly, pearson correlation analyses demonstrated that after normalization of hypercholesterolemia, the size of advanced lesions was also highly correlated with collagen (r=0.91, p=0.0002, n=16) and necrotic core (r=0.80, p=0.0003, n=16) content, but not correlated with macrophage content (r=0.39, p=0.14, n=16).

Effects of dietary lipid lowering on inflammatory cells in the adventitia of mice with early and advanced lesions

Adventitial inflammatory cells also play a critical role in the progression of atherosclerotic lesions [13]. The response of inflammatory cells in the adventitia to dietary lipid lowering might thus influence the progression of established lesions. Therefore, total and degranulated mast cells, neutrophils, and T cells in the adventitia underlying the early and advanced lesions were analyzed following WTD withdrawal. Upon dietary lipid lowering, total and degranulated mast cells in the adventitia of early lesions increased 2.1-fold (p<0.05 vs C and W1) and 3.8-fold (p<0.05), respectively at 3 weeks and remained stably high (total: 2.8-fold, p<0.01 vs C; degranulated: 3.9-fold, p<0.05 vs C) at 6 weeks (Figure 6B&C). Degranulated mast cells can recruit neutrophils by secretion of KC, the murine ortholog of IL-8 [14]. In line, also a significant increase of neutrophils (1.9-fold, p<0.01 vs C; 1.2-fold, p<0.05 vs W1) was observed in the adventitia of early lesions at 3 weeks after WTD withdrawal. Unexpectedly, at 6 weeks, neutrophils declined 2-fold to approximately the levels at baseline (p<0.05 vs W3) (Figure 6D). As compared to early lesions, the adventitia of advanced lesions contained more total (1.7-fold, p=0.0251) and degranulated (5.2-fold, p=0.0072) mast cells and neutrophils (3.6-fold, p=0.0008) before the diet switch. While total mast cells did not change in the adventitia of advanced lesion after the WTD withdrawal, dietary lipid lowering did lead to a reduction of degranulated mast cells (3.1-fold, p<0.001 vs C) after 1 week on chow, which remained stably low during the remainder of the experiment (Figure 6B and 6C). Neutrophils in the adventitia of advanced lesions declined in time (6.8-fold, p<0.001, W6 vs C) to the baseline levels of early lesions at 6 weeks on chow (Figure 6D).

As an important component of adaptive immunity, T cells are critical for the progression rather than initiation of atherosclerosis [15]. In line, 16-fold more T cells (p=0.0004) were observed in the adventitia of advanced lesions as compared to early lesions. As shown in Figure 6E, T cells in the adventitia of early lesions increased approximately 2-fold (p<0.01 vs C) in the first week after the WTD withdrawal and did not significantly change afterwards, while a 1.7-fold (p<0.05 vs W1) reduction of T cells was detected in the adventitia of advanced lesions from 1 to 3 weeks, which remained low (1.9-fold, p<0.01 vs W1) at 6 weeks on chow. The amount of T cells in the adventitia of advanced lesions, however, remained 7.9-fold (p=0.0003) higher as compared to the levels in early lesions at 6 weeks on chow.
Figure 6. Effect of dietary lipid lowering on inflammatory cells in the adventitia of early and advanced lesions. (A) Representative photomicrographs of mast cells (200x), degranulated mast cells (arrow, 200x), neutrophils (100x) and T cells (100x) in the adventitia. Bar graphs showing the dynamic changes of total mast cells (B), degranulated mast cells (C), neutrophils (D) and T cells (E) in the adventitia of early (closed circles) and advanced (open circles) lesions after the switch of WTD to chow diet. Values are means±SEM (n=8). Statistically significant difference *P<0.05, **P<0.01, and ***P<0.001 versus group C; #P<0.05, ##P<0.01, and ###P<0.01 versus group W1; $P<0.05 versus group W3.

Effects of dietary lipid lowering on oxidative stress in early and advanced lesions

To study the effect of dietary lipid lowering on oxidative stress in the established lesions, the amount of nitrotyrosine and 8-hydroxy-2’-deoxyguanosine (8-OHdG) in the lesions were quantified after the diet switch. Although the plasma cholesterol decreased after 6 weeks on chow, the amount of nitrotyrosine and 8-OHdG in the early lesions was significantly increased 17-fold (p=0.009) and 3.3-fold (p=0.043), respectively in the early lesions (Supplementary Figure S3A). In contrast, the oxidative stress was reduced in advanced lesions as evidenced by the reduction of nitrotyrosine (13-fold, p=0.005) and 8-OHdG (5.0-fold, p=0.0003) (Supplementary Figure S3B).

Effects of dietary lipid lowering on the established atherosclerotic lesion development in the coronary artery and aortic arch

The lesion development in the coronary artery and aortic arch was next analyzed. In line with the fact that the aortic root is the most susceptible site for atherosclerosis in murine
models, no lesion formation was visible in the coronary artery of LDLr KO mice after 5 weeks WTD feeding. Even after 9 weeks on WTD, only 2 out of 8 mice displayed very small lesions (lesion size: $5.6 \times 10^3 \mu m^2$ and $7.2 \times 10^3 \mu m^2$). Moreover, LDLr KO mice did not develop any atherosclerotic lesions in the aortic arch after 5 weeks on WTD (data not shown). After 9-week WTD feeding, macrophage-rich fatty streak lesions had formed in the aortic arch with a mean lesion area of $86.5\pm16.4 \times 10^3 \mu m^2$ (Supplementary Figure S4A). The ratio of collagen to macrophages in the aortic arch after 9 weeks on WTD ($0.053\pm0.021$) was similar to the established early lesions in the aortic root ($0.051\pm0.015$) after 5 weeks WTD feeding. No necrotic core was visible in these established lesions of the aortic arch (Supplementary Figure S4D). After the first week on the chow, no significant change was found in the lesion size ($97.8\pm13.4 \times 10^3 \mu m^2$) and composition (Supplementary Figure S4). Three weeks chow diet feeding led to a ~2.0-fold ($p<0.05$ vs C and W1) and ~7.5-fold ($p<0.01$ vs C and W1) increase in size and collagen content, respectively (Supplementary Figure S4A and C). In addition, a small necrotic core became evident. However, the mean size was only $2.5 \times 10^3 \mu m^2$ ($p<0.05$ vs C and W1) (Supplementary Figure S4D). The lesion increased further in size (1.5-fold, $p<0.05$ vs W3; ~3-fold, $p<0.001$ vs C and W1) and collagen content (2.4-fold, $p<0.001$ vs W3; ~20-fold vs C and W1) after an additional 3 weeks on chow, i.e. total 6 weeks on chow (Supplementary Figure S4A and C). Also, an enlargement of the necrotic core was observed (5.5-fold, $p<0.001$ vs W3). The ratio of collagen to necrotic core was thereby reduced 5.2-fold to $5.8\pm1.3$ ($p<0.05$ vs W3) (Supplementary Figure S4D). Meanwhile, dietary lipid lowering did not significantly affect the macrophage content, although a tendency to a similar profile was observed as established early lesions in the aortic root (Supplementary Figure S4B). Taken together, dietary lipid lowering did lead to similar changes on early lesions in both aortic root and aortic arch.

Discussion

The present study for the first time investigated the dynamic remodeling of established early and advanced atherosclerotic lesions upon dietary lipid lowering. Strikingly, both established early and advanced atherosclerotic lesions continued to progress after the withdrawal of dietary lipids, which was strongly correlated with the induction of collagen and necrotic core content of the lesions. However, after normalization of hypercholesterolemia, early lesions exhibited a proinflammatory phenotype while advanced lesions became less inflammatory. The clear differences in the response of early and advanced lesions to dietary lipid lowering indicate that the severity of the lesions affected their dynamic response to lipid lowering.

Dietary lipid lowering, as expected, induced a decrease in plasma and hepatic cholesterol levels. However, both established early and advanced lesions in LDLr KO mice could further increase in size when the plasma cholesterol levels of the animals was normalized to around 220 mg/dL, i.e. lower than 300 mg/dL that is prerequisite for initiation of lesion development [16]. This finding indicates that the plasma cholesterol levels required for lesion progression are lower than those for lesion initiation. This might also explain why progression of atherosclerotic lesions can occur in patients with normal LDL-C levels (< 90 mg/dL) after lipid lowering treatment with statins [3]. In line, a robust reduction of the plasma LDL-C levels (<70 mg/dL) is required to halt the progression of established atherosclerotic lesions in humans [3, 4]. However, it should be noted that some patients cannot reach this threshold level of LDL-C even after intensive lipid lowering treatment. Combined therapy that targets multiple pathogenic mechanisms involved in lesion progression is thus needed for efficient treatment of this group of patients.
Consistent with previous findings in rabbits [17] and miniature pigs [18], dietary lipid lowering also led to massive collagen accumulation in the lesion of LDLr KO mice. Importantly, we observed that the collagen content was highly correlated with the size of both early and advanced lesions from 3 to 6 weeks after normalization of hypercholesterolemia. Despite being important for providing mechanical stability to the lesion, excessive collagen may also stimulate lesion development by serving as a depot for proatherogenic molecules (modified lipoproteins, growth factors and glycation end-products) and by promoting foam cell formation [19]. Moreover, massive collagen accumulation might obstruct the access of healthy phagocytic cells to clear apoptotic cells in the lesions, thereby leading to the increased necrotic core formation. Necrotic core areas are sites of active inflammation and oxidative stress [20, 21], which could thus further promote the progression of lesions. In agreement, we found that necrotic core formation increased in time upon dietary lipid lowering. It should thus be carefully investigated if inhibition of collagen synthesis in combination with lipid lowering might be a potential therapeutic strategy for the treatment of atherosclerosis.

Recruitment of monocytes into atherogenic foci is central to the initiation and progression of atherosclerosis. Hypercholesterolemia induces Ly6C\textsuperscript{high} monocytosis [22, 23], subsequently contributing to atherogenesis [12]. Reduction of plasma cholesterol levels by statins could reduce Ly6C\textsuperscript{high} monocytosis [22]. However, the anti-inflammatory effects of statins beyond cholesterol lowering could also contribute to these alterations. In the present study, we showed that cholesterol lowering alone by dietary lipid withdrawal could not only decrease circulating monocytes, but also normalize the increased levels of Ly6C\textsuperscript{high} monocytes in LDLr KO mice. Of note, these effects were mainly observed at 3 weeks after the diet switch. Therefore, both early and advanced lesions continued to accumulate macrophages in the first week after dietary lipid lowering, probably as a result of monocyte recruitment from the circulation. Swirski et al demonstrated that apart from the bone marrow, spleen is another reservoir for a large number of monocytes [24]. These splenic monocytes can be recruited to inflammatory sites. Interestingly, reduced monocytes in the subcapsular red pulp of spleen was also observed after 1 week on chow (unpublished data), indicating that splenic monocytes might account for the increased macrophage accumulation in lesions within the first week after the diet switch. However, more experiments remain to be done to test this hypothesis. From 1 to 3 weeks after the diet switch, dietary lipid lowering reduced the macrophage content of both early and advanced lesions. Meanwhile, the size of macrophage foam cells and necrotic core was not significantly changed. Moreover, the rate of apoptosis was either very low in the early lesions or remain stable in the advanced lesions, we thus speculate that the reduction of macrophages is mainly the consequence of their emigration. Emigration of monocyte-derived foam cells from atherosclerotic lesions and regression of established atherosclerotic lesions has been elegantly shown by transplantation of a region of the aorta with atherosclerotic lesions from hypercholesterolemic apoE KO mice to normocholesterolemic wildtype mice [25]. From 1 to 3 weeks after WTD withdrawal, the lesion size was not increased in early lesions and even reduced in advanced lesions, indicating that dietary lipid lowering can inhibit the progression and/or induce the regression of established lesions. However, despite the reduced Ly6C\textsuperscript{high} monocytosis upon dietary lipid lowering, macrophage accumulation in the early lesions was enhanced again from 3 to 6 weeks after the diet switch. Interestingly, the macrophage area was highly correlated with the lesion size of early lesions but not of advanced lesions at this time point after WTD withdrawal. In agreement, using bone marrow transplantation we recently demonstrated that the growth of established early lesions under hypercholesterolemia is mainly caused by the continuous recruitment of monocyte-derived cells, while this process...
Stage-specific remodeling of atherosclerotic lesions is largely impaired in established advanced lesions [26]. Thus, inhibition of monocyte infiltration combined with lipid lowering might be a more efficient therapeutic strategy for the treatment of established early lesions than advanced lesions.

Another important finding is that early and advanced lesions showed a different inflammatory phenotype after normalization of hypercholesterolemia. Early lesions became proinflammatory, as evidenced by a decreased ratio of collagen to necrotic core, an elevated neutrophil content, and increased oxidative stress, while advanced lesions became less inflammatory. Zernecke et al demonstrated that an expansion of circulating neutrophils leads to more neutrophil infiltration into lesions [27]. However, dietary lipid lowering did reduce the levels of circulating neutrophils. This thus cannot contribute to the increased neutrophil infiltration in the lesions upon dietary lipid lowering. Moreover, in line with previous study [28], the area of necrosis did not correlate with neutrophil accumulation. Actually, almost no neutrophil was found in close proximity of the necrotic core in the atherosclerotic lesion. Interestingly, quantification of inflammatory cells in the adventitia of early lesions revealed increased numbers of degranulated mast cells. Activation of mast cells not only recruits monocytes, neutrophils and other mast cells by secreting their respective chemoattractant MCP-1, KC and eotaxin [14, 29], but also promotes macrophage apoptosis, probably thereby leading to the augmented buildup of necrotic core material [30]. In line, the enhanced infiltration of monocytes and neutrophils into the lesions, the increased recruitment of mast cells into the adventitia, and a dramatic enlargement of necrotic core were observed upon progression of the early lesions after normalization of hypercholesterolemia. Increased macrophages and neutrophils in early lesions could generate more reactive oxygen species through NADPH oxidase and myeloperoxidase [31], thereby leading to the enhanced oxidative stress. Therefore, the proinflammatory phenotype of early lesions might be the consequence of mast cell activation in the adventitia. Importantly, Sun et al recently demonstrated that mast cells promote atherogenesis by promoting the secretion of the proinflammatory cytokine IL-6 and IFN-γ [32]. Also, the activation of mast cells is involved in induction of rupture of advanced lesions [30]. Targeting mast cell activation might thus be a promising strategy for combination with lipid lowering therapies for treatment of established atherosclerotic lesions.

We recognize the limitations of the current study. Dietary lipid lowering might not be as effective as the use of statins in the treatment of atherosclerosis, due to their many pleiotropic effects beyond their cholesterol lowering capacity [33]. Also mice have no cholesterol ester transport protein (CETP) while humans do [34]. Moreover, in contrast to humans, LDLr KO used in our study developed atherosclerosis at the aortic root and descending aorta rather than coronary artery. In addition, mice seldom show signs of myocardial infarction or stroke [35] as murine atherosclerotic lesions are not prone to plaque rupture. Therefore, it is clear that findings based on mouse models must be carefully evaluated in larger animal models and in humans.

In summary, our study provides important insights into the dynamic remodeling of established atherosclerotic lesions upon dietary lipid lowering. Progression of established atherosclerotic lesions after dietary lipid lowering indicates that more effective therapeutic strategies are needed for the treatment of atherosclerosis. The dynamics of lesion remodeling upon dietary lipid lowering are complex and depend on the stage of atherosclerotic lesion development. Establishment of stage-specific therapeutic protocols might thus improve the eventual outcome. However, the requirement of stage-specific therapy for atherosclerosis calls for the development of better molecular imaging tools to characterize lesion compositions in vivo.
Chapter 8

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References

10. Bennett MR. The atherosclerotic plaque was not built in a day: the dynamic nature of plaque progression and instability. Heart Metab. 2007; 36:5-7.
Stage-specific remodeling of atherosclerotic lesions


Supplementary Figures

**Supplementary Figure S1.** Effect of dietary cholesterol lowering on lipoprotein distribution of total cholesterol (TC). LDLr-/- mice were fed WTD for 5 weeks to induce the lesion formation. Thereafter, the animals were switched to regular chow and euthanized at 0 (C), 1 (W1), 3 (W3), and 6 weeks after the diet switch. 30 μL aliquot of serum was fractionated by a Pharmacia Smart column and TC of each fraction was determined. Fractions 3 to 6 represent VLDL, fractions 8 to 12 represent LDL, and fractions 16 to 20 represent HDL. Values represent the mean±SEM of 8 mice.

**Supplementary Figure S2.** Effect of dietary cholesterol lowering on the cell apoptosis in the established lesions. LDLr-/- mice were fed WTD for 5 and 9 weeks to induce the lesion formation. Thereafter, the animals were switched to regular chow and euthanized at 0 (C), 1 (W1), 3 (W3), and 6 weeks after the diet switch. Apoptosis is expressed as the percentage TUNEL-positive to total nuclei in the atherosclerotic lesions. Values represent the mean±SEM of 8 mice. Statistically significant difference *P<0.05 and ***P<0.001 versus group C; †P<0.05 and ‡‡P<0.001 versus group W1; ‡P<0.05 versus group W3.
Supplementary Figure S3. Effect of dietary cholesterol lowering on oxidative stress in the established lesions. LDLr-/- mice were fed WTD for 5 and 9 weeks to induce the lesion formation. Thereafter, the animals were switched to regular chow and euthanized at 0 (C), 1 (W1), 3 (W3), and 6 weeks after the diet switch. Oxidative stress is expressed as the mount of nitrotyrosine and 8-hydroxy-2-deoxyguanosine (oh8dG) in the atherosclerotic lesions. Values represent the mean±SEM of 5 mice. Statistically significant difference *P<0.05, **P<0.01, and ***P<0.001 versus group C.

Supplementary Figure S4. Effect of dietary cholesterol lowering on the development of established lesions in the aortic arch. LDLr-/- mice were fed WTD for 9 weeks to induce the lesion formation. Thereafter, the animals were switched to regular chow and euthanized at 0 (C), 1 (W1), 3 (W3), and 6 weeks after the diet switch. Mean lesion area (A) and macrophage (B), collagen (C) and necrotic core (D) content were quantified as mentioned in the materials and methods. Values represent the mean±SEM of 8 mice. Statistically significant difference *P<0.05, **P<0.01, and ***P<0.001 versus group C; **P<0.05, ***P<0.01, and ****P<0.001 versus group W1; $P<0.05$ and $$$P<0.001 versus group W3.