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Abstract—Intimal hyperplasia after vascular injury is usually studied in animal models with healthy, normocholesterolemic animals. Here, we assess the effect of diet-induced hypercholesterolemia on the induction of intimal hyperplasia in ApoE*3Leiden mice. A nonconstrictive polyethylene cuff was placed around the femoral artery of ApoE3*Leiden mice fed a highly cholesterol-rich diet, a mildly cholesterol-rich diet, or a chow diet for 4 weeks. Diets were continued after cuff placement until euthanization. At several time points (1 to 14 days), mice were euthanized and the intimal hyperplasia in the cuffed arteries was analyzed. In mice fed a chow diet, a 2- to 4-cell-layer–thick intima, predominantly consisting of α smooth muscle cell actin–positive cells, was observed after 14 days. A mildly cholesterol-rich diet (mean plasma-cholesterol level, 10.5 mmol/L) resulted in a 2.7-fold increase of total intimal area, and a highly cholesterol-rich diet (mean plasma cholesterol level 28.6 mmol/L), in a 7.8-fold increase. In the high-cholesterol group, the intima consisted predominantly of lipid-loaded foam cells and α smooth muscle cell actin–positive cells. Foam cell accumulation could be observed by as early as 3 days, resulting in a near-total occlusion of the lumen after 14 days. Hypercholesterolemia resulted in a rapid, cholesterol-dependent induction of foam cell–rich intimal hyperplasia in cuffed femoral arteries of ApoE*3Leiden mice. In conclusion, the present data show that the combination of a local (cuff placement) and a systemic (hypercholesterolemic) risk factor of atherosclerosis results in a rapid induction (within 14 days) of atherosclerotic-like lesions in ApoE*3Leiden mice. **(*Circ Res*. 2000;87:248-253.)**

Key Words: atherosclerosis ■ foam cell accumulation ■ transgenic mice ■ intimal hyperplasia

Intimal thickening is an early and essential stage in the development of atherosclerotic lesions.[1] In patients undergoing a venous bypass graft procedure, accelerated vein graft atheroma together with intimal hyperplasia is frequently observed.[2] In these patients, a prolonged history of hypercholesterolemia is often present. Therefore, studying the effect of hypercholesterolemia on intimal hyperplasia after a vascular intervention in an atherosclerotic animal model is indispensable to clarifying the underlying mechanisms of accelerated atherosclerosis in patients after a vascular intervention. The process of intimal thickening has been extensively evaluated in animal models using several techniques. Experimental intimal hyperplasia can be induced by balloon denudation of the endothelium,[3] by ligation of the vessel,[4] by placement of a rigid polyethylene cuff,[5,6] by placement of a flexible silicone collar,[7,8] or by electrical injury.[9] Intimal thickening induced by these techniques results from excessive accumulation of smooth muscle cells and deposition of extracellular matrix in the intimal layer of the vessel wall.[3,10,11] Recently, it has been reported that placement of a nonconstrictive collar around an arterial segment in mice results in a reproducible, concentric intimal hyperplasia within 21 days.[6] This intimal hyperplasia predominantly consists of α smooth muscle cell actin–positive cells, as is also reported for rabbits by several other groups.[6,8,12] However, the species (mice, rats, and rabbits) used in these studies were predominantly young, healthy, and normocholesterolemic animals.

Mice are one of the most useful experimental animals because of their small size, easy maintenance, short breeding time, and the possibilities for carrying out advanced genetic studies such as transgenesis and gene targeting. Transgenic technologies have provided numerous different murine strains to study hyperlipidemia and atherosclerosis. The ApoE*3Leiden transgenic mice[13] develop diet-dependent hyperlipidemia and are highly susceptible to diet-induced atherosclerosis. In these mice, when fed a mildly cholesterol-rich, high-fat diet, early fatty-streak formation (stage I, American Heart Association [AHA] classification,[14]) can be observed in the aortic arch after 3 months of treatment, and late complex atherosclerotic lesions, consisting of plaques with a necrotic core and a fibrous cap (stages IV and V, AHA classification[14]), can be registered after 3 to 6 months of

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feeding a severe cholesterol-rich diet. This animal model, in which the atherosclerotic lesions have many features in common with human atherosclerotic lesions,[15,16] is currently considered as one of the animal models of atherosclerosis closest to that occurring in humans.

In the current study, this animal model of cholesterol-induced atherosclerosis has been used to evaluate the effect of plasma cholesterol on the development of intimal thickening by placing a polyethylene cuff around the femoral artery of ApoE*3Leiden mice.

We found that the combination of systemic and local risk factors resulted in an accelerated formation of atherosclerotic-like lesions in mice. After the placement of a nonconstrictive cuff, a plasma cholesterol level–dependent intimal thickening was induced within 14 days. This intimal thickening started within 3 days after cuff placement with monocyte adhesion and foam cell accumulation and progressed until near-total occlusion of the lumen after 14 days.

Materials and Methods

Mice

All experiments were approved by the committee on animal welfare of TNO (The Organization for Applied Scientific Research). Specific pathogen–free transgenic ApoE*3Leiden mice were crossbred for 18 generations with C57BL/6 mice. Male animals, age 8 to 10 weeks, were allocated randomly to 1 of the 3 experimental diets on the basis of age and litter.

Diets

During the experimental period, animals were fed a chow diet; a cholesterol-enriched high-fat diet containing 0.5% cholate to improve intestinal cholesterol uptake and suppress bile acid synthesis, both leading to increased plasma cholesterol levels (high fat and cholate–enriched [HFC] diet 0.5%: casein 20%, choline chloride 1%, methionine 0.2%, cocoa butter 15%, cholate 0.5%, cholesterol 1%, sucrose 40.5%, cornstarch 10%, corn-oil 1%, cellulose 5.1%, and mineral mixture 5.1%); or a cholesterol-enriched, high-fat diet containing 0.05% cholate (HFC 0.05%: casein 20%, choline chloride 1%, methionine 0.2%, cocoa butter 15%, cholate 0.05%, cholesterol 1%, sucrose 40.5%, cornstarch 10%, corn oil 1%, cellulose 4.7%, and mineral mixture 5.1%) 4 weeks before operation and continued after operation.

Placement of Cuff

After 4 weeks of chow, HFC 0.05%, or HFC 0.5%, mice were anesthetized with Hypnorm (fentanyl citrate 0.315 mg/mL/fluanisone 10 mg/mL; Bayer, 25 mg/kg IP) and Dormicum (midazolam 5 mg/mL Roche, 25 mg/kg IP). The right femoral artery was dissected from its surroundings. A nonconstrictive polyethylene cuff (Portex, 0.40-mm inner diameter, 0.80-mm outer diameter, and 1.5-mm length) was placed loosely around the right femoral artery.[6]

Lipids and Lipoprotein Analysis

Blood samples were taken under general anesthesia from the tail vein at the time of operation and euthanization. Total plasma cholesterol (Boehringer Mannheim GmbH, kit 236691) and triglyceride (Sigma Diagnostics, kit 337-B) concentrations were measured enzymatically using commercially available kits.

Histological Assessment of Intimal Lesions

At euthanization, mice were anesthetized with Hypnorm/Dormicum. The thorax was opened, and mild pressure-perfusion (100 mm Hg) with 3.7% formaldehyde in 0.9% NaCl (wt/vol) for 10 minutes was performed by cardiac puncture. After perfusion, the femoral artery was harvested, fixed overnight in 3.7% formaldehyde in PBS, and paraffin-embedded. Serial cross sections (5 μm thick) were used throughout the entire length of the cuffed femoral artery for histological analysis. Cryosections were made of 2 mice in each condition. All samples were routinely stained with hematoxylin-phloxine-saffron (HPS). Weigert's elastin staining was used to visualize elastic laminae. Smooth muscle cells were visualized with α-smooth muscle cell actin staining (Boehringer Mannheim), and Mac-3 (Accurate Chemical) macrophage staining was used to detect monocytes/macrophages. Lipid deposition was visualized with Oil-red-O (Boehringer Mannheim) staining. Anti–platelet-endothelial cell adhesion molecule-1 (PECAM)-1 antibodies (Sigma) were used as endothelial cell marker.

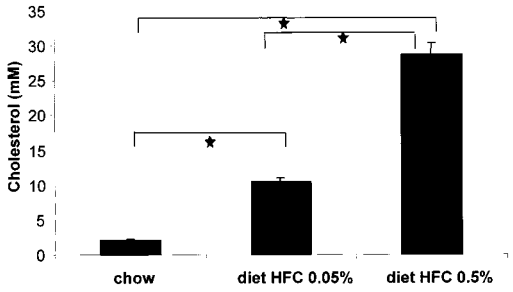

Figure 1. Plasma cholesterol and triglyceride levels at euthanization in ApoE*3Leiden mice fed a chow diet (n=10), HFC 0.05% diet (n=10), and HFC 0.5% diet (n=10) for 6 weeks. *$P<0.001$. No significant changes in triglyceride levels were detected.

Quantification of Intimal Lesions in Sections of Cuffed Femoral Artery

Ten equally spaced cross sections were used in all mice to quantify intimal lesions. Using image analysis software (Leica), total cross-sectional medial area was measured between the external and internal elastic lamina; total cross-sectional intimal area was measured between the endothelial cell monolayer and the internal elastic lamina.

Statistics

All data are presented as mean±SEM. Overall comparisons between groups were performed with the Kruskal-Wallis test. If a significant difference was found, groups were compared with their respective controls using Mann-Whitney rank-sum tests. A value of $P<0.05$ was regarded as significant.

Results

Plasma Lipid Levels

Body weights were monitored on the day of the placement of the cuff and at euthanization. No significant changes in body weights were registered in any of the animals. Plasma cholesterol and triglyceride concentrations at euthanization are shown in Figure 1. Compared with the standard mouse diet (chow), both the HFC 0.05% and HFC 0.5% diets increased plasma cholesterol concentrations significantly, whereas serum triglyceride concentrations were decreased, as described earlier.[17,18] The distribution of lipids among the different lipoprotein classes in ApoE3*Leiden mice on a HFC 0.5% diet are highly comparable with the human situation. A shift in lipoprotein profile from the HDL-sized fraction toward the VLDL/LDL-sized fractions, especially when fed HFC 0.5% diet, was observed as reported previously.[15,18]

Time Course of Development of Intimal Lesions

To study the effect of hypercholesterolemia on the development of intimal hyperplasia in time, 5 mice on the chow diet

and 5 mice on the HFC 0.5% diet were killed at respectively 1, 3, 7, 10, and 14 days after placement of the cuff. In the animals on a chow diet, α smooth muscle cell actin–positive cells invaded in the intimal region within 7 days after the placement of the cuff. After 14 days, the intimal lesion area was ≈2 to 4 cell layers thick. This is in accordance with the intimal lesions observed in C57BL/6 and SV 129 mice after cuff placement on a chow diet published by Moroi et al[6] and also found by us (data not shown). However, in the mice fed the HFC 0.5% diet, as early as 3 days after the placement of the cuff, intimal thickening was observed on the luminal side of the inner elastic lamina. This intimal thickening progressed in time until near-total occlusion of the lumen was achieved after 14 days (Figure 2). In these hypercholesterolemic animals, monocyte adhesion to the endothelial layer was observed as early as 1 day after the placement of the cuff. After 3 days, monocyte adhesion became more distinct and the first foam cells could be detected. Seven days after the cuff placement, macrophages were present in the media, in addition to the intimal lesion (Figure 3A). Endothelial cells, monitored by PECAM-1 expression, were observed 3 and 7 days after the cuff placement (Figure 3B).

Characterization and Quantification of Intimal Lesions

To characterize the effect of hypercholesterolemia on intimal hyperplasia, 20 male ApoE*3Leiden mice were randomized in 2 groups. One group (n=10) was fed a chow diet, and 1 group (n=10) was fed a HFC 0.5% diet, 4 weeks before placement of the cuff to obtain stable plasma cholesterol levels. Fourteen days after placement of the cuff, light microscopy of transverse sections through the cuffed femoral artery revealed that in the mice on a chow diet a thickening of the intimal region occurred (Figure 4), whereas proximal and distal sections of the cuffed femoral artery possessed normal histology (data not shown). This intimal thickening was ≈2 to 4 cell layers thick and consisted predominantly of α smooth muscle cell actin–positive cells and to a lesser extent monocytes/macrophages. An intact endothelial layer and inner elastic lamina was observed in the arteries 14 days after cuff placement. No foam cells were detected in any of the sections taken from the cuffed arteries of normocholesterolemic animals at 14 days.

In the group of animals fed a high-cholesterol, cholate-containing diet (HFC 0.5%) for 4 weeks, atherosclerotic-like lesions, predominantly consisting of a massive accumulation of macrophage foam cells in the intimal region, were observed 14 days after the placement of the cuff. At the luminal side of this foam cell accumulation, deposition of α smooth muscle cell actin–positive cells partly on top of the macrophages could be observed as a sign of the start of lesion cap formation. The deposition of foam cells and smooth muscle cells occurred underneath an apparently intact endothelial layer and on an intact inner elastic lamina. These spindle-like macrophage foam cells were lipid-filled and primarily present in the intimal region but also to a lesser extent in the medial region.

The abundant accumulation of macrophage foam cells and smooth muscle cells in the mice fed HFC 0.5% resulted in a

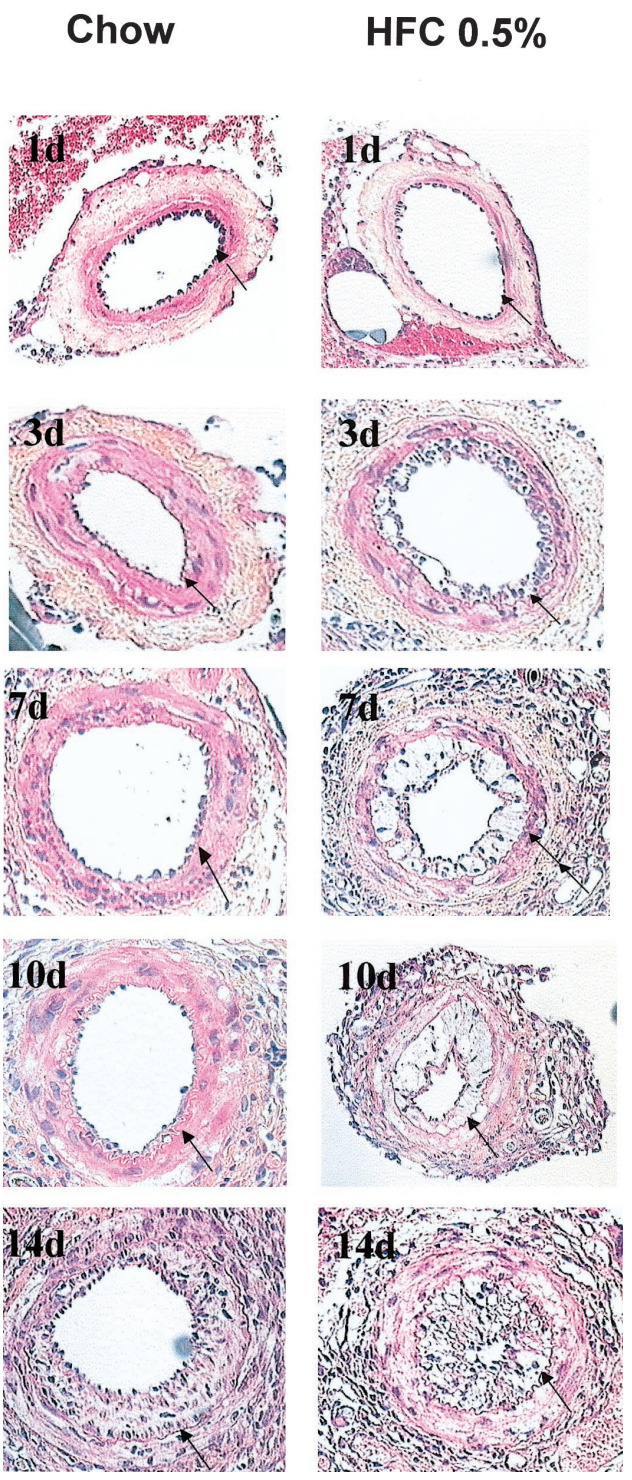

Figure 2. Cross section of murine femoral artery on several time points (1, 3, 7, 10, and 14 days) after placement of cuff in mice on a chow diet and HFC 0.5% diet. In mice on a chow diet, the first signs of neointima formation were observed 7 days after placement of the cuff. After 14 days, intimal hyperplasia was 2 to 4 cell layers thick. In mice on the HFC 0.5% diet, however, cell adhesion was observed on the luminal side of the inner elastic lamina 1 day after cuff placement. After 3 days, foam cell deposition on the inner elastic lamina was detectable in mice on a HFC 0.5% diet. Seven days after cuff placement in mice on a HFC 0.5% diet, foam cell accumulation in the intimal lesion progressed, and foam cells were observed in the media. Foam cell accumulation progressed in time until near-total occlusion of the lumen 14 days after cuff placement in mice on a HFC 0.5% diet. Magnification ×10.

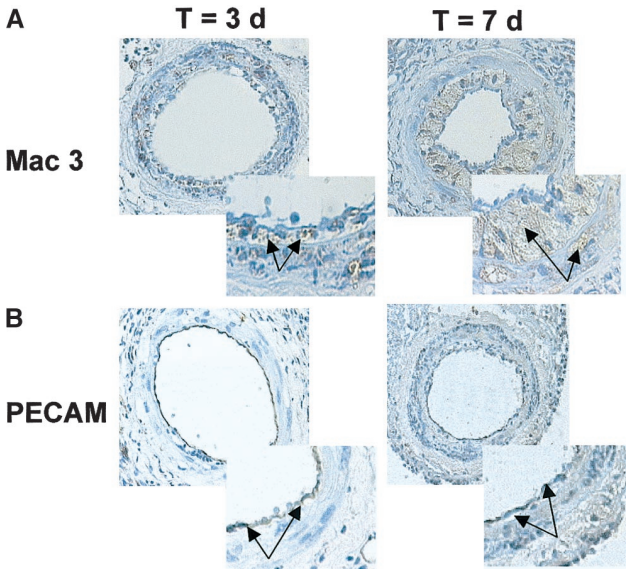

Figure 3. A, Cross sections of murine femoral artery on 3 and 7 days after placement of cuff in mice on a HFC 0.5% diet stained for monocyte/macrophage using the monoclonal antibody Mac-3. Monocyte adhesion is detectable on the luminal side of the inner elastic lamina as early as 3 days after cuff placement. Profound macrophage infiltration both in the neointima and in the media was detectable after 7 days. B, Cross section of murine femoral artery on 3 and 7 days after placement of cuff in mice on a HFC 0.5% diet stained for PECAM. Magnification ×10; insets ×20. Positive-staining cells are indicated by arrows.

profound luminal narrowing compared with mice on a chow diet. A near-total occlusion of the lumen was frequently observed in the mice on HFC 0.5% 14 days after placement of the cuff. However, a total occlusion due to intimal hyperplasia or thrombosis never occurred in any of the animals.

Correlation Between Plasma Cholesterol Levels and Intimal Lesion Development

To evaluate the correlation between serum cholesterol levels and the development of atherosclerotic lesions in the cuffed femoral artery, 3 groups of 9 ApoE*3Leiden mice received chow diet, HFC 0.05% diet, or HFC 0.5% diet 4 weeks before the cuff placement. At the time of the placement of the cuff, these mice had a mean serum cholesterol level of 2.3±1.2, 13±5.6, and 32±3.2 mmol/L, respectively. Quantification of total intimal areas of 3 groups of ApoE*3Leiden mice revealed a significant 2.3-fold increase in mean total intimal area in mice fed HFC 0.05% diet ($P<0.001$) and a 7.8-fold increase in mice fed an HFC 0.5% diet ($P<0.001$) compared with mice on a chow diet. Additionally, significant differences in intima/media ratio and percentage luminal stenoses were observed between mice on the 3 different diets (Figure 5). Correlation between plasma cholesterol levels and intimal thickening of each mouse is depicted in Figure 6.

Discussion

In this report, we describe the effect of hypercholesterolemia on intimal lesion formation after placement of a nonocclusive cuff around the femoral artery in ApoE*3Leiden mice. In accordance with the results previously described by Moroi et

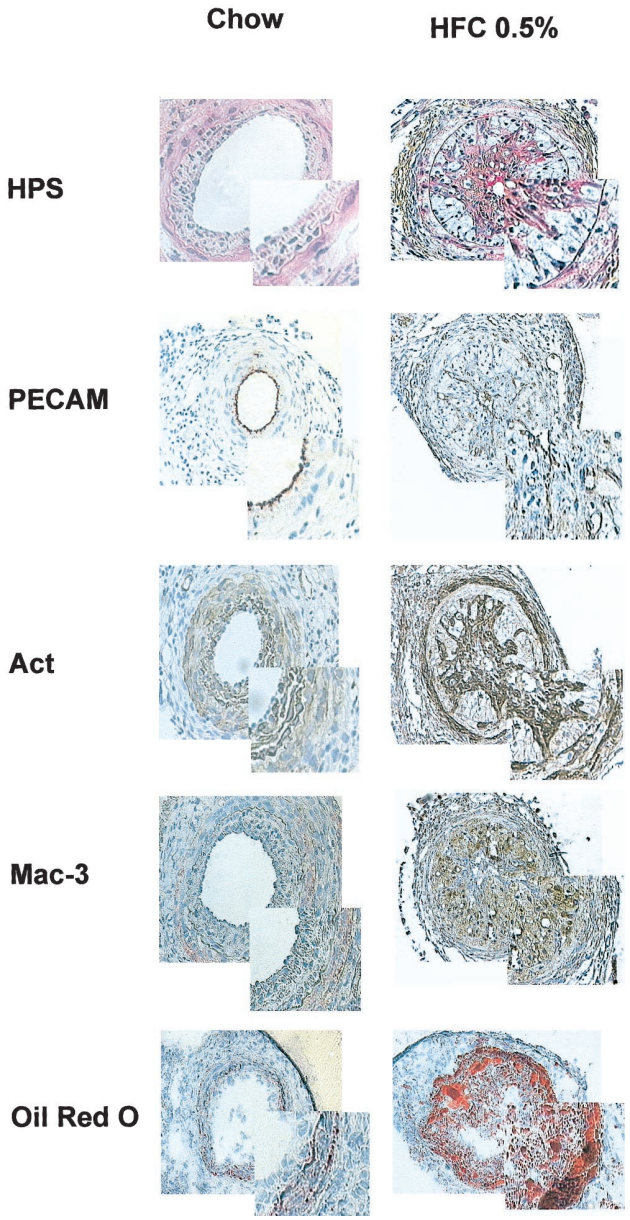

Figure 4. Cross section of the murine femoral artery 14 days after placement of cuff in mice on a chow diet and HFC 0.5% diet; shown is HPS staining. In mice on a chow diet, a multiple-cell layer–thick intimal hyperplasia is observed. In mice on a HFC 0.5% diet, significant increase in intimal hyperplasia is observed; shown is PECAM staining for endothelial cells. In both mice on a chow diet and mice on the HFC 0.5% diet, endothelial cells are present at the luminal side of the intimal lesion; shown is α smooth muscle cell actin staining for smooth muscle cells. Intimal hyperplasia in mice on a chow diet predominantly consists of α smooth muscle cell–positive cells. In mice fed a HFC 0.5% diet, smooth muscle cells occur mainly on the luminal side of the foam cell accumulation; shown is Mac-3 staining for macrophages. Intimal lesions of mice on the HFC 0.5% diet predominantly consist of lipid-loaded foam cell macrophages. Macrophages are also present in granulation tissue within the cuff of both mice on a chow diet, as in mice on a HFC 0.5% diet; shown is Oil red O lipid staining. Abundant lipid deposition in foam cells in mice on a HFC 0.5% diet, both in the intimal lesion as well as in the media. Arrow indicates inner elastic lamina; polyethylene cuff is visible as a white band. Magnification ×10; insets ×20.

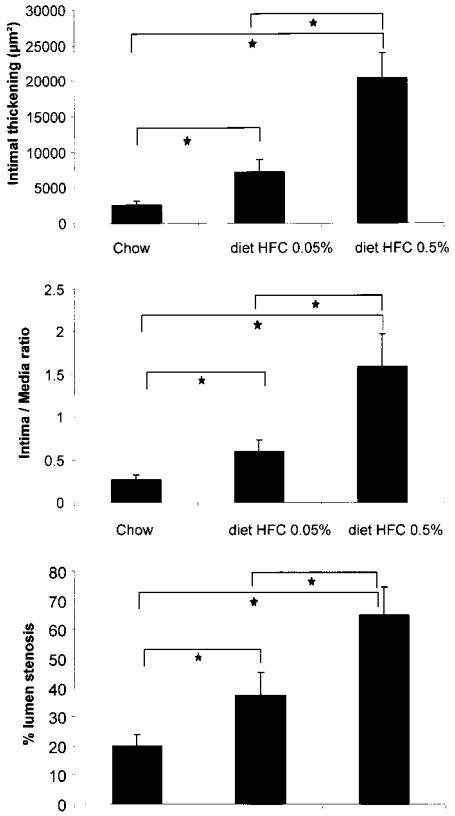

Figure 5. Total intimal area (top), intima/media ratio (middle), and percentage luminal stenosis (bottom) of ApoE*3Leiden mice fed HFC 0.1% diet (n=9) and HFC 0.5% diet (n=9) vs chow diet (n=9) 14 days after cuff placement. Total intimal area was quantified by image analysis using 10 serial sections in each cuffed artery and expressed in μm^2 (mean±SEM). *$P<0.001$.

al[6] in C57BL/6 and SV129 mice, placement of a nonconstrictive polyethylene cuff around the femoral artery of ApoE*3Leiden mice on a chow diet resulted in intimal lesions predominantly consisting of α smooth muscle actin–positive cells only. In the lesions of these normocholesterolemic animals, no foam cell macrophages were registered. Placing a cuff on ApoE*3Leiden mice on a high-cholesterol diet, however, resulted in an extremely fast and profound induction of atherosclerotic-like lesions. These lesions predominantly consisted of lipid-loaded foam cells and to a lesser extent of α smooth muscle cell actin–positive cells. The accumulation of spindle-like lipid-filled macrophage-derived foam cells on the luminal side of the inner elastic lamina is a typical feature of early human, postintervention atherosclerosis.[14]

Two weeks after the placement of the cuff, the atherosclerotic-like lesions that developed in mice on a high-cholesterol diet were abundant and resulted in a near-total occlusion of the lumen. The total area of these lesions was significantly larger (7.8-fold) than lesions developed in mice on a chow diet. Quantification of the total intimal area of mice on different diets (chow and mildly and highly cholesterol rich) revealed that there is a correlation between plasma cholesterol and total intimal growth. This correlation has been described earlier in transgenic mice developing atherosclerosis spontaneously on a high-cholesterol diet after several months.[15]

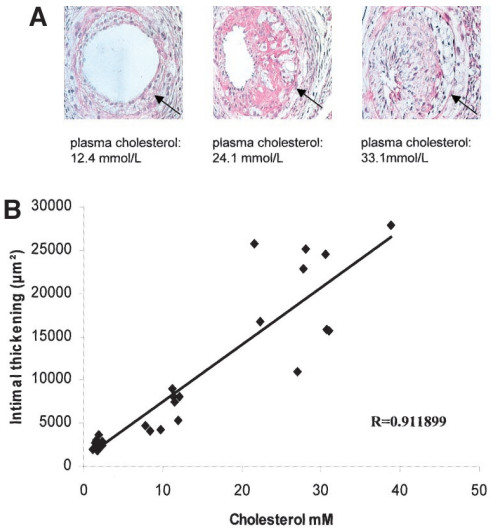

Figure 6. A, Representative cross sections of murine femoral arteries of mice with different plasma cholesterol levels (HPS staining). B, Correlation of total intimal area and serum cholesterol level in ApoE*3Leiden mice fed a chow diet (n=18), HFC 0.05% diet (n=9), and HFC 0.5% diet (n=9). Total intimal area was calculated by image analysis using 10 serial sections in each cuffed artery.

The development of the atherosclerotic-like lesions started directly after the placement of the cuff as demonstrated by monocyte adhesion, which was already present after 1 day. The first deposition of lipid-filled foam cells on the luminal side of the inner elastic lamina was observed as early as 3 days after the placement of the cuff.

Both phenomena illustrate the extremely fast initiation of the atherosclerotic process. Also, the total area of these lesions after 14 days is far more extensive than lesions developed spontaneously in transgenic mice after a prolonged period on a high-cholesterol HFC 0.5% diet without the placement of a cuff.[16]

The exact mechanism by which collar-induced intimal thickening is triggered is still unclear. The disturbance of the laminar blood flow, vascular damage, production of cytokines by the granulation tissue, and elimination of transmural flow by the cuff may account for the intimal thickening.[19] In the cuffed artery, shear and wall stress is altered, resulting in endothelial damage and activation.[7] This leads to intimal hyperplasia predominantly consisting of smooth muscle cells in normocholesterolemic animals. In hypercholesterolemic animals, however, endothelial activation is accompanied by the accumulation of lipids in the infiltrating monocytes, resulting in excessive foam cell formation. Consistent with this observation is the fact that the predilection sites of spontaneous atherosclerosis in transgenic mice (aortic valve, aortic branch, and bifurcation sites) are sites with disturbance in laminar flow and subsequent damage to the endothelial layer.

Human-like atherosclerotic lesions develop spontaneously in ApoE*3Leiden mice when fed an atherogenic diet for several months. However, this process of atherosclerotic lesion development is rather slow, and it takes ≈3 to 6 months on a severe cholesterol diet before type IV and type V lesions (nomenclature of AHA classification used by Stary

et al[14]) can be observed.[15,16] Furthermore, the exact lesion location and onset of atherosclerosis in time is unknown. These aspects imply that the analysis of the mechanisms of atherosclerosis and the evaluation of antiatherosclerotic pharmacological and nonpharmacological strategies in this mouse model is a time-consuming process. In addition, because atherosclerotic lesions, which develop spontaneously in transgenic mice, are frequently located in the central part of the arterial tree, local antiatherosclerotic strategies are difficult to apply. Therefore, the induction of atherosclerotic-like lesions within several weeks, at a predefined, easily accessible site in the arterial tree, with a known onset in time, would be preferable for studying factors causing the early onset of atherosclerotic-like lesions and for assessing the effect of systemic and local antiatherosclerotic therapies. As shown, the cuff model for accelerated atherosclerotic-like lesions is suitable for studying the early steps in atherosclerotic plaque formation. Moreover, the accelerated atherosclerosis observed in human vein grafts, with less organized structures lacking typical features such as the fibrous cap and necrotic core, shows high morphological resemblance to the atherosclerotic lesions observed in this model.[20]

The approach of induction of intimal thickening by placement of a nonocclusive perivascular cuff in mice, as described above, has several characteristics in common with collar-induced intimal thickening in rabbits. As in rabbits, in mice, intima formation occurs without endothelial cell denudation. However, the intimal lesions in ApoE*3Leiden mice on a high-cholesterol diet predominantly consist of lipid-loaded foam cells, whereas collar-induced lesions in Watanabe rabbits mainly consist of α smooth muscle cell actin–positive cells.[8]

In conclusion, in the present study we demonstrated that, by the placement of a nonconstrictive polyethylene cuff around a femoral artery of ApoE*3Leiden mice, highly reproducible, accelerated, atherosclerotic-like lesions can be induced. These lesions display human characteristics, especially in the early phase, and are dependent on plasma cholesterol level. This accelerated, diet-dependent induction of atherosclerotic-like lesions is of value in studying the mechanisms of accelerated atherosclerosis.

Acknowledgments

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