Atherosclerosis in APOE*3-Leiden Transgenic Mice
From Proliferative to Atheromatous Stage

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Background—This study documents (1) the progression of atherosclerosis along the entire arterial tree in APOE*3-Leiden mice after 1, 4, 6, 9, and 12 months of a high-fat/high-cholesterol (HFC) diet and (2) the amount and phenotype of DNA-synthesizing and apoptotic cells in different lesion types after 6 months of HFC diet.

Methods and Results—Diet duration was correlated with a craniocaudal progression of lesion development and with an increase in severity of the lesion. Typically, the lesions contained smooth muscle cells, macrophages, and T lymphocytes and were covered by an intact endothelium. Whereas DNA synthesis (BrdU uptake) was usually elevated in type II lesions (8.6±0.8% versus 1.0±0.2% in the nondiseased arterial wall; P<0.05), apoptosis was found primarily in advanced lesions (type IV, 1.3±0.1% and type V, 1.2±0.2% versus 0.04±0.04% in the nondiseased arterial wall [P<0.05]). Cell phenotyping revealed that the majority of DNA synthesis and apoptosis was confined to the macrophage-derived foam cell (68.6±3.0% and 82.2±4.6%, respectively).

Conclusions—This study shows that in APOE*3-Leiden mice, duration of an HFC diet is associated with (1) a craniocaudal progression of lesion development and (2) an increased complexity of atherosclerotic lesions. Furthermore, DNA synthesis is predominant in early lesions, whereas apoptosis is present mainly in more advanced lesions. Both parameters of cell turnover are confined primarily to the macrophage-derived foam cell. (Circulation. 1999;99:276-283.)

Key Words: atherosclerosis ■ genetics ■ apoptosis

One of the mutant apoE genes that is associated with familial dysbetalipoproteinemia in humans is the APOE*3-Leiden gene.1,2 To evaluate the phenotype in more detail, this gene has been introduced in mice, the APOE*3-Leiden mouse.3,4

The data published so far show that APOE*3-Leiden mice exhibit severe hypercholesterolemia when they are fed a high-fat/high-cholesterol (HFC) diet. However, atherosclerosis in these mice has been described only after relatively short periods of cholesterol feeding.3,5,6 To date, no detailed description of lesion morphology including data on cell turnover is available. Also, data on the progression of lesions along the entire arterial tree and long-term observations on the progression of plaque extent and complexity are lacking. This study was initiated to provide these data.

Methods

Mice

Transgenic mice expressing the human APOE*3-Leiden gene were generated and bred with C57Bl/6J females as described earlier.3,4 For the present study, 33 male and 27 female mice of the F10 generation were used.

Diet

Before entry into the study, animals were kept on standard mouse chow (SRM-A, Hope Farms). At the age of 37±0.6 weeks (mean±SEM), the animals were put on a diet containing 15% cacao butter, 0.5% cholate, 1% cholesterol, 40.5% sucrose, 10% corn starch, 1% corn oil, and 4.7% cellulose (Hope Farms)7 for 1 (n=4), 4 (n=9), 6 (n=12), 9 (n=12), and 12 (n=12) months. Control APOE*3-Leiden (n=6) and C57Bl/6J (n=6) mice received standard mouse chow for 9 months. To label DNA-synthesizing cells, 6 transgenic animals of the 6-month diet group received 5′-bromo-2′-deoxyuridine (BrdU, Serva; infusion rate, 13 mg · kg⁻¹ · d⁻¹) by an osmotic minipump (Alzet 2001, Alza Corp) for the 7 days before they were euthanized. Pumps were placed subcutaneously between the shoulder blades under ether anesthesia. All mice were housed under standard conditions. The study was approved by the institutional committee for the welfare of laboratory animals of the University of Maastricht.

Tissue Handling

After completion of the diet according to protocol, each mouse was anesthetized with xylazine 0.0025 mL/g and ketamine 0.001 mL/g. Blood (0.5 to 1 mL) was collected from the caval vein for the assessment of lipid profile, and the arterial tree was perfused with 0.9% NaCl (3 minutes) and 10% phosphate-buffered formalin (pH 7.4, 3 minutes), both containing 20% nitroglcerin, through a...
catheter into the left ventricular apex. Subsequently, the complete arterial tree was taken out and fixed overnight in 10% phosphate-buffered formalin.

All arterial segments indicated in Figure 1 were processed and embedded in paraffin, and 4-μm sections were stained with hematoxylin and eosin. Atherosclerotic lesions were classified according to the guidelines given by the American Heart Association (AHA) by 1 investigator (E.L.), who was blinded to presence or absence as well as duration of diet and to genotype.

Assessment of Lipid Profile
Total serum cholesterol and triglycerides (but not free glycerol) were measured as described previously.1

Immunohistochemistry

Study 1: Diet-Dependent Lesion Progression
Because of its size and because all lesion types also developed in the aortic arch, we concentrated the immunohistochemical analyses on this segment. Parallel sections of the aortic arch were immunolabeled with either ED-19 for the detection of macrophage-derived foam cells, CD-3 (CD-3, monoclonal, 1:200, Dako A0452) for the detection of T lymphocytes, α-smooth muscle actin (α-smooth muscle actin–labeled secondary antibodies and 3,3′-diaminobenzidine (DAB) as a chromogen. Sections were counterstained with hematoxylin and mounted with coverslips. Negative controls included omission of TdT from the labeling mixture. Tonsils were used as a positive control.

Cell Phenotypes. Parallel sections of atherosclerotic lesions of the 6 APOE*3-Leiden mice were immunolabeled with ED-1, α-smooth muscle actin, CD-3, and factor VIII as described above.

Cell Counting
Tissue sections were investigated by light microscopy at ×400 magnification with a standard field size. All nuclei of the respective lesion type were counted. Cells containing dark nuclear BrdU staining were considered to be DNA-synthesizing cells. Apoptotic nuclei were defined as TUNEL-positive nuclei in cells that showed morphological features of apoptotic cell death (cell shrinkage, aggregation of chromatin into dense masses, cell fragmentation).12

The labeling index, defined as the total number of positive cells divided by the total number of cells, was calculated for the entire atherosclerotic lesion, including the underlying media. This was performed separately for the BrdU- and TUNEL-stained sections. Labeling indices were also determined in different sites of the lesion: the underlying media, the endothelial coverage, the shoulder region, the lipid core, and the fibrous cap. Furthermore, the distribution of the different immunophenotypes of BrdU- or TUNEL-positive cells was quantified. The immunophenotype of 89.1 ± 1.4% of the DNA-synthesizing cells and 97.3 ± 2.2% of the apoptotic cells could be determined by the above-described panel of antibodies (CD3, ASMA, FVIII, ED-1).

All measurements were performed by 1 investigator (E.L.). Intraobserver variation was <10%.

Statistical Analysis

Study 1: Diet-Dependent Lesion Progression
Data are expressed as mean±SEM or as median values (AHA classification). All experimental groups were compared with the APOE*3-Leiden mice on normal chow. Atherosclerotic lesions of male and female mice in the different diet groups were compared by a nonparametric Mann-Whitney U test. Because no significance was found, atherosclerotic lesions of male and female mice were pooled. Cholesterol, triglyceride, and apoE levels were analyzed by the Kruskal-Wallis test, and Student’s t test was used for comparisons between the groups.

Study 2: Cell Turnover in Atherosclerotic Lesions
Data are expressed as mean±SEM.

To test whether lesions of the same type in different mice and lesions of the same type at different sites of the arterial tree were comparable, a 1-way ANOVA was performed on both parameters. This was performed for both BrdU- and TUNEL-stained sections. Because no significant difference was found, lesions of the different mice and at the different sites of the arterial tree were assumed to be comparable.

Subsequently, a Mann-Whitney U test was used for comparisons between the lesion types. In all tests, the level of statistical significance was assumed to be at P<0.05.

Results

Study 1: Diet-Dependent Lesion Progression
Cholesterol, Triglyceride, and ApoE Levels
In the APOE*3-Leiden mice on the HFC diet, all values except triglyceride levels after 1 month of HFC diet were significantly higher than in both control groups (P<0.05) (Table 1). Human apoE plasma levels were elevated in all transgenic animals, confirming the expression of the APOE*3-Leiden gene.
Atherosclerotic Lesions in APOE*3-Leiden Transgenic Mice

Atherosclerotic lesions developed in the aorta and large vessels in all APOE*3-Leiden mice on an HFC diet (Figure 1). Lesions developed in the proximal coronary arteries (Figure 2A), the aortic root, the aortic arch and its main branch points (Figure 2B), the thoracic aorta, the abdominal aorta, the renal artery branch points (Figure 2C), the abdominal aorta bifurcation, and the iliac artery bifurcations. Almost all lesion types were observed: initial lesions with isolated macrophage foam cells (type I), fatty streaks with mainly intracellular lipid accumulation (type II, Figure 3A), and intermediate lesions with type II changes and small extracellular lipid pools (type III). Also, more advanced lesions could be detected, such as an atheroma with type II changes and a core of extracellular lipid (type IV, Figures 2B and 3D) as well as fibroatheromata with a lipid core and a fibrotic layer (Figure 2A and 2C), or with multiple lipid cores and fibrotic layers (type Va), or mainly calcific (type Vb, Figures 2D and 3E) or fibrotic (type Vc) fibroatheromata. Type VI lesions with plaque rupture, thrombus formation, and hemorrhage were not observed.

In the APOE*3-Leiden mice on normal chow, only initial type I and II lesions were observed. Wild-type mice on normal chow did not develop atherosclerosis.

Immunophenotype

Macrophages were not present in the normal media but were found in the media and intima of all lesion types (Figure 3A). In advanced lesion types, lipid-filled macrophages were present primarily in the shoulder region of the lesion. T lymphocytes were present primarily in advanced lesion types (IV to Vb), especially in the shoulder region and fibrous cap (Figure 3B).

Both the media of nondiseased vessel segments and the nondiseased media below an atherosclerotic lesion showed

<table>
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<tr>
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<th>Cholesterol, mmol/L</th>
<th>Triglycerides, mmol/L</th>
<th>Human ApoE, g/L</th>
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<td>APOE*3, 1m HFC (n=4)</td>
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<td>0.4±0.1</td>
<td>0.4±0.06*</td>
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<td>APOE*3, 6m HFC (n=12)</td>
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<td>2.3±0.2*</td>
<td>0.9±0.11*</td>
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<tr>
<td>APOE*3, 9m HFC (n=12)</td>
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<td>2.0±0.3*</td>
<td>1.0±0.10*</td>
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<tr>
<td>APOE*3, 12m HFC (n=12)</td>
<td>30.6±3.9*</td>
<td>2.4±0.3*</td>
<td>0.7±0.08*</td>
</tr>
<tr>
<td>APOE*3, 9m NC (n=6)</td>
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<td>0.2±0.05</td>
<td>0.1±0.06</td>
</tr>
<tr>
<td>C57BL/6J, 9m NC (n=6)</td>
<td>0.8±0.11*</td>
<td>0.1±0.04</td>
<td>0.0±0.0</td>
</tr>
</tbody>
</table>

Values are mean±SEM. APOE*3 indicates APOE*3-Leiden transgenic mice; C57BL/6J, C57BL/6J (wild-type) mice; m, months; and NC, normal chow. *P<0.05 vs APOE*3-Leiden mice on normal chow.
no desmin-positive vascular smooth muscle cells (VSMCs) (Figure 3C and 3D). However, desmin-positive VSMCs were present in the intima and fibrous cap of advanced lesions (Figure 3D). All atherosclerotic lesions contained α-smooth muscle actin–positive VSMCs, both in the non-diseased media below the lesion and in the lesion itself (Figure 3E). They were most numerous in the fibrous cap of advanced lesion types. The endothelial coverage of the lesions remained intact, even in very advanced lesions (Figure 3F).

**Effect of Diet Duration on Lesion Progression**

The variability in the development of atherosclerotic lesions between mice in 1 diet group was remarkably low, and diet duration seemed to predict lesion type and site (data not shown). After 1 month of HFC diet, initial type II lesions were observed only in the aortic root, the aortic arch and its main branches, the carotid artery bifurcations, and the thoracic aorta. Below the diaphragm, no lesions were observed.

After 4 months of HFC diet, initial type II lesions were present in the coronary arteries. Advanced atherosclerotic

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**Figure 3.** Immunohistochemistry of atherosclerotic lesions in APOE*3-Leiden mice with different antibodies. A, ED-1; B, CD-3; C, desmin; D, desmin; E, ASMA; and F, factor VIII. L indicates lumen.
lesions (type IV) with a core of extracellular lipid were present in the aortic root, the aortic arch and its main branch points, and the right carotid artery bifurcation. Below the diaphragm, only initial lesions (type I and II) with foam cells in the subendothelium were observed (Figure 1).

After 6 months of HFC, advanced type IV lesions had developed in the coronary arteries (Figure 2A). Furthermore, type V lesions with a fibrous cap and a lipid core were present in the aortic arch and its main branches (Figure 2B). Type IV lesions had also developed in the abdominal aorta and renal artery branch points.

After a diet period of 9 months, severe calcification of the atherosclerotic lesions (type Vb) was observed in the aortic arch and its main branch points (Figure 2D). Type V lesions were present in the right carotid artery bifurcation. Type IV lesions had developed in the thoracic aorta, in the abdominal aorta, and at the renal artery branch points. However, in the abdominal aorta and the iliac artery bifurcations, only type II lesions could be observed.

After 12 months of HFC diet, lesions in the aortic arch showed calcification (type Vb), and type V lesions were observed at both the right and left carotid artery bifurcations. Moreover, 5 of 12 mice showed calcifications in their coronary arteries. Furthermore, at the abdominal aorta bifurcation and the iliac artery bifurcations, advanced lesions (type IV) had developed (Figure 1).

**Study 2: Cell Turnover in Atherosclerotic Lesions**

In total, 135 lesions were investigated throughout the arterial tree of the 6 APOE*3-Leiden mice fed an HFC diet for 6 months. BrdU- and TUNEL-positive nuclei were counted in all lesion types except in type I lesions, because the total number of nuclei in this lesion type was too small. Thirty nondiseased arterial segments served as controls.

**DNA Synthesis**

In all lesion types, DNA synthesis was significantly elevated compared with the nondiseased arterial wall (Figure 4). The highest BrdU labeling was found in type II (fatty streak) lesions (8.6 ± 0.8% versus 1.0 ± 0.2% in the nondiseased arterial wall; *P* < 0.05) (Figure 4A). With progression of severity of the lesion, DNA synthesis decreased to 5.8 ± 0.7% in complex type V lesions (*P* < 0.05 versus type II).

Not only the level but also the site of DNA synthesis changed with progression of the lesion (Figure 4D). In type II lesions, most DNA-synthesizing cells were found in the media (77.9% of all DNA-synthesizing cells), whereas in type III, IV, and V lesions, only a small amount of DNA-synthesizing cells was found in the media (17.1%, 14.1%, and 12.1%, respectively, *P* < 0.05). With the development of a shoulder region in type III and IV lesions, DNA synthesis shifted to that region (32.9% and 41.0% of all DNA-synthesizing cells, respectively) as well as to the endothelial cells covering the lesion (30.0% and 32.1%, respectively). The percentage of DNA synthesis of endothelial cells covering type III and IV lesions, was significantly increased compared with both type II lesions and the nondiseased arterial wall (*P* < 0.05). When atherosclerosis had progressed to advanced type V lesions with a fibrous cap and a lipid core, DNA-synthesizing cells were located in the shoulder region (36.2%) and fibrous cap (29.3%), whereas the fraction of DNA-synthesizing cells of endothelial cells covering the fibrous cap showed a significant decrease to 12.1% compared with type IV lesions (*P* < 0.05). DNA synthesis in the lipid core of type IV and V lesions was low (12.8% and 12.1% of all DNA-synthesizing cells, respectively).

Subsequent immunolabeling (Figure 4) showed that 68.6 ± 3.0% of the DNA-synthesizing cells were macrophage-derived foam cells, 14.5 ± 2.5% VSMCs, 1.3 ± 0.3% T lymphocytes, and 5.3 ± 1.2% endothelial cells of the endothelial coverage. In all lesion types, the majority of DNA-synthesizing cells were foam cell–derived macrophages, but the ratio of macrophage-derived foam cells to VSMCs decreased with lesion development. In early type II lesions, this ratio was 76.9, whereas in complex type V lesions, this ratio had decreased to 3.7 (*P* < 0.05).

**Apoptosis**

The distribution of apoptotic nuclei in the different lesion types differed from the distribution of DNA-synthesizing nuclei (Figure 5). In type II and III lesions, the apoptotic labeling index was not different from the apoptotic labeling index in the nondiseased arterial wall. The percentage of apoptotic nuclei was elevated only in type IV and V lesions (1.3 ± 0.1% in type IV and 1.2 ± 0.2% in type V versus 0.04 ± 0.04% in the nondiseased arterial wall). The majority of apoptotic nuclei was located in the lipid core of these type IV and V lesions (81.9% and 79.5% of all apoptotic cells, respectively). Furthermore, only low levels of apoptosis were found in the fibrous cap (9.0% of all apoptotic cells).

Cell phenotyping revealed that the majority of apoptosis was confined to the macrophage-derived foam cells (82.2 ± 4.6%) (Figure 5). Apoptosis in other cell types was rarely observed.

**Discussion**

One of the most important steps in atherogenesis is the transition of a fatty streak into an atherosclerotic plaque. In the present study, it was demonstrated that fatty streaks were characterized by DNA synthesis with no detectable apoptosis. These lesions progress toward atherosclerotic plaques, which were characterized by the presence of both DNA synthesis and apoptosis. This finding is comparable to the classic studies of Virchow, who described atherosclerosis as a formative process, starting with “proliferation” and terminating with “fatty degeneration.”

So far, few human data on DNA synthesis during the entire process of atherogenesis are available. Orekhov et al report peak levels of DNA synthesis in lipid-rich lesions, whereas DNA synthesis in fibrous lesions is much lower. The majority of data on DNA synthesis in human atherosclerosis are confined to advanced lesions only. They show low levels of DNA synthesis (0% to 2%), but this level is significantly higher than in the nondiseased arterial wall.

One study on DNA synthesis in early fatty-streak lesions also reports low values of DNA synthesis (0% to 2%). DNA synthesis in these human lesions is confined primarily to either the macrophage-derived foam cell or the...
VSMC. As in human in vitro studies and rabbit models, the APOE*3-Leiden mouse exhibits a DNA-synthesis peak in early lesion types. DNA synthesis in advanced lesion types is low, which is in accordance with the low levels of DNA synthesis found in advanced human atheromata. Interestingly, we observed 2 kinds of type II lesions. The early type II lesion contained macrophage-derived foam cells that were located only in the media, whereas the late type II lesion contained macrophage-derived foam cells in the intima. We defined them as type IIa and IIb, respectively. In the early type IIa lesions, DNA synthesis was significantly elevated compared with the late type IIb lesions (10.3 ± 0.8% in type IIa versus 6.0 ± 1.3% in type IIb). In both lesion types, the majority of DNA-synthesizing cells were macrophage-derived foam cells (91.1% and 94.2%, respectively). The fact that the intima of a nondiseased vessel in the mouse consists of only a very small subendothelial layer may explain our observation that early lesions contain medial macrophage-derived foam cells, whereas in advanced lesions, these macrophages are present in the intima. Thus, it seems that the first migrating macrophage-derived foam cells are transported from the blood into the most luminal layer of the media. With the accumulation of increased numbers of macrophage-derived foam cells (type IIb through Vb), a neointima develops.

Enhanced rates of apoptotic cell death have also been observed in advanced human atherosclerosis. In a recent study, we found that fatty streak lesions are characterized by very low levels of apoptosis, whereas advanced atherosclerotic lesions showed remarkable levels of cell death. In human atheromata, the apoptotic labeling index varies from 0% to 40%, and apoptotic nuclei are present primarily in the...
lipid core and regions adjacent to the lipid core, but also in the fibrous cap. The majority of apoptotic nuclei have been found in macrophages and T lymphocytes. Also, in APOE*3-Leiden mice, apoptosis was confined to the advanced stages, albeit at fairly low levels. Moreover, apoptosis in the fibrous cap was a rare phenomenon. Whether this phenomenon can be an explanation for the apparent plaque stability observed in these mice remains to be investigated.

One of the major differences of atherosclerosis in the APOE*3-Leiden mouse with human atherosclerosis is the absence of plaque rupture, thrombus formation, and/or hemorrhage in the atherosclerotic plaque. We have no explanation for the apparent stability of the atherosclerotic plaques in the APOE*3-Leiden mouse. One of the possibilities is that the morphologically intact endothelial layer that covers all lesion types in the APOE*3-Leiden mouse prevents lesions from rupturing. Another explanation is the low level of apoptosis in the fibrous cap in these mice.

The data presented here may have clinical implications. It is known that treatment with HMG-CoA reductase inhibitors can slow the progression of atherosclerosis. One of the mechanisms that have been described is the inhibition of DNA synthesis in both VSMCs and macrophages. Our data imply that these agents may be most successful in slowing the progression of atherosclerosis when they are applied early in the disease process, when DNA synthesis is usually elevated. Conversely, it may be more interesting to look at agents that modulate apoptosis. Because apoptosis is a late event in atherogenesis, inhibition of apoptosis may both slow the progression of atherosclerosis and be effective in preventing the conversion from stable into unstable atherosclerotic lesions.

In the present article, we demonstrate that APOE*3-Leiden mice show a highly reproducible diet- and time-dependent cranio-caudal progression of atherosclerosis extent and plaque complexity. We found a peak in DNA synthesis in early...
lesions and a peak in apoptosis in late lesions, both which are confined to macrophage-derived foam cells. Diet dependency, predilection site, plaque composition, and cell turnover make the APOE*3-Leiden mouse a suitable model to study different aspects of early atherogenesis and the evaluation of pharmacological and nonpharmacological interventions.

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References
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