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The fetal origin of adult atherosclerosis : a study in ApoE and Ldlr mouse models

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The Fetal Origin of Adult Atherosclerosis

A Study in ApoE and Ldlr Mouse Models

Fanneke Elizabeth Alkemade

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A Study in ApoE and Ldlr Mouse Models

Fanneke Elizabeth Alkemade

Thesis Leiden University Medical Center

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The Fetal Origin of Adult Atherosclerosis

A Study in ApoE and Ldlr Mouse Models

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*The pessimist sees difficulty in every opportunity.
The optimist sees the opportunity in every difficulty.*

- Sir Winston Churchill -

Voor mijn ouders

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Chapter 1

General Introduction

Introduction

Cardiovascular Diseases

In The Netherlands, the contribution of cardiovascular diseases to the total mortality is 32% (Centraal Bureau voor de Statistiek (CBS), Mortality in 2006). Consequently, cardiovascular diseases are still the number one cause of death. Atherosclerosis is the underlying cause for more than 50% of cases. Already more than 50 years ago, in 1951, fatty streaks were generally considered to be the earliest atherosclerotic lesions.¹ These lesions were mainly found in aortas in childhood and adolescence.²⁻⁴ In 1975, Sinzinger and colleagues reported the existence of lipid deposits even in fetal aortas.⁵ Although the process of atherosclerosis appears to start already early in life, decades of lesion progression follow until eventual atherosclerosis-associated events occur, such as myocardial infarction and ischemic stroke.

Atherosclerosis is a complex multifactorial process. Many risk factors that contribute to atherosclerosis have been identified. They can be divided into uncontrollable and controllable, genetic and environmental factors. Important risk factors are hypercholesterolemia, hypertension, diabetes mellitus, obesity, lack of physical activity, diet, alcohol consumption, smoking, genetically inherited traits, gender, age and family history.

Fetal Origin of Cardiovascular Disease

The “fetal origins hypothesis” postulated by Barker⁶ proposes that cardiovascular disease risk is at least partially determined by the intrauterine environment created by the mother during pregnancy. He stated that an embryo is capable of adjustment to unfavorable aspects of the maternal environment during development. As a result, the intrauterine environment becomes beneficial to the embryo and chances of survival increase. However, when the adult environment differs from the fetal environment, these adaptations may be detrimental for health in adult life.

A number of studies have reported an association between intrauterine growth restriction and increased cardiovascular disease risk in adult life.⁶⁻⁸ A unique study setting in this context is the Dutch Hunger Winter Families Study. During the harsh winter of 1944-1945, in the final year of the Second World War, there was severe food shortage in the Western part of The Netherlands. The cohort comprises individuals who were prenatally exposed to famine and controls that were not. Data revealed increased cardiovascular disease risk at adult age in the former group, especially in those exposed during early gestation.^{9,10}

Besides intrauterine growth restriction, more and more evidence is provided that maternal hypercholesterolemia during pregnancy may have adverse effects on cardiovascular disease risk in the offspring. Cholesterol is an essential component for adequate development of the embryo. It is an integral part of cell membranes, for example lipid rafts. In the third trimester of human pregnancy, in which fetal growth rate and subsequent fetal requirements are enhanced, the majority of mothers develop gestational hypercholesterolemia.^{11,12} Hence, gestational hypercholesterolemia is generally considered to be a natural phenomenon not directly posing a risk for cardiovascular disease in both mother and progeny. However, in 1997, Napoli and colleagues demonstrated that maternal hypercholesterolemia during pregnancy accelerated and aggravated fatty streak formation in fetal aortas.¹³ An extended study in neonates and young children demonstrated more rapid progression of atherosclerosis in children from hypercholesterolemic mothers compared with those from normocholesterolemic mothers.¹⁴ Based on these data one can speculate that offspring from hypercholesterolemic mothers acquire a long-term susceptibility for atherosclerosis and are at increased risk for myocardial infarction or ischemic stroke in adult life.

Epigenetic Programming of Cardiovascular Disease

The “fetal origins hypothesis” suggests that an adverse intrauterine environment induces changes that persist into adulthood. This so-called intrauterine programming is not achieved by alterations in the DNA sequence itself, but by epigenetic mechanisms. The word epigenetics was already introduced by Waddington in 1942¹⁵ and was used to describe cell differentiation. Later it was defined as the study of the mechanisms of temporal and spatial control of gene activity during the development of complex organisms.¹⁶ Nowadays, epigenetics is preferably defined as the processes that through chromatin remodeling alter gene expression patterns within a cell without changing the nucleotide sequence. The mostly stable epigenetic changes can be transmitted to the daughter cells. DNA methylation of CpG islands¹⁷⁻¹⁹ and covalent post-translational histone modifications²⁰ are key players in the regulation of the transcription machinery.

A clear definition of epigenetics does not mean that the interpretation is simple. Epigenetic programming namely means different things to different people. Cancer researchers study the role of epigenetics in the context of dysregulation or defects of the normal epigenome, the overall epigenetic state of a cell. Aberrant epigenetic modifications of the chromatin surrounding the gene, for example loss or gain of methylation, are considered to be the cause of altered expression and subsequent development of disease.²¹⁻²³ Geneticists on the other hand, study the role of epigenetic mechanisms in imprinting of heritable diseases. Epigenetics also

comprises the effects of environmental factors on the epigenome. It has been reported that maternal behavior and care during the lactation period determine the extent of DNA methylation and histone acetylation on the glucocorticoid receptor in the rat offspring, thereby programming the stress response and social behavior.²⁴⁻²⁸

Intrauterine programming can be established by a number of, mainly environmental, cues (reviewed by Fowden and colleagues²⁹). As mentioned earlier, maternal nutrition is very important during pregnancy to supply for the fetal needs. A shift in nutritional balance may induce programming. Physiological stress and dysregulation of the endocrine system have been reported to affect blood pressure, glucose tolerance, and the stress response in the offspring.^{30,31} Finally, environmental toxins and substances such as alcohol and nicotine may influence intrauterine programming.^{32,33} The role of epigenetic mechanisms in intrauterine programming becomes more and more apparent. Recently, it has been demonstrated that exposure to famine induces epigenetic changes in the IGF2 gene that persist into adulthood.³⁴

Aim of This Thesis

Although data indicate that an adverse intrauterine environment comprised of environmental atherosclerotic risk factors such as hypercholesterolemia, oxidative stress and inflammation, may induce accelerated and aggravated atherosclerotic lesion formation in fetuses, it is not known whether this actually results in increased cardiovascular disease development in adult life. Execution of a study in humans on the long-term consequences of intrauterine exposure is nearly impossible. Extensive genetic variation between individuals in combination with many environmental factors, prospective follow-up for decades and possible ethical objections are potential biases and have to be taken into account. To overcome this, often mouse models of atherosclerosis are used. The most frequently utilized mouse model is the apolipoprotein E-deficient (apoE^{-/-}) mouse strain.^{35,36} ApoE^{-/-} mice spontaneously develop hypercholesterolemia on a regular chow diet. In addition, atherosclerotic lesions similar to that found in humans develop throughout the arterial tree. A second mouse model is the low-density lipoprotein receptor-deficient (Ldlr^{-/-}) mouse strain.^{37,38} In this model, Western-type diet feeding is used to induce hypercholesterolemia and subsequent atherosclerosis development. In general, the atherosclerotic lesions in the murine arterial tree are initially characterized as fatty streaks containing lipid cores and cholesterol crystals.³⁹ In more advanced lesions calcification can be detected, though limited.³⁹ The aforementioned suggests that the apoE^{-/-} mouse model is a perfect model for mimicking human atherosclerosis and cardiovascular disease. The opposite is true. Although the pathogenesis of atherosclerosis in the aorta can be studied, Coleman

and colleagues³⁹ never found lesions in the coronary artery. As a result, mice will not suffer from myocardial infarction. In addition, plaque rupture, an important feature that underlies human myocardial infarction can not be studied.^{40,41} Vulnerable plaques and subsequent rupture can be detected in the brachiocephalic artery of mice. However, there is no consensus on whether or not this observed vulnerability meets the criteria set for the human situation.⁴¹

Since both the apoE^{-/-} and Ldlr^{-/-} strain are good models for studying early pathogenesis of atherosclerosis, we used them in the experiments described in this thesis. Early atherosclerotic lesion development is studied in the aorta and neointima formation in the carotid artery. We generated heterozygous offspring, apoE^{+/-} and Ldlr^{+/-} mice. In comparison to apoE^{-/-} and Ldlr^{-/-} mice, heterozygous mice are athero-resistant. In this way, we were able to examine the effects of environmental risk factors. In addition, we addressed the challenging question how an adverse maternal environment can be translated into an increased atherosclerosis susceptibility of the offspring. Which maternal signals affect the offspring and what epigenetic mechanisms are involved to predispose the developing embryo or fetus to increased atherosclerosis susceptibility?

Chapter Outline

In **Chapter 2** we demonstrate that intrauterine exposure to maternal atherosclerotic risk factors associated with apoE-deficiency induces susceptibility for cardiovascular disease in apoE^{+/-} offspring that persists into adult life.

Chapter 3 shows that maternal apoE-deficiency is sufficient to allow collar-induced neointima formation in apoE^{+/-} offspring, even without cholesterol feeding, sustaining the hypothesis that the intrauterine environment poses a major risk factor for adult cardiovascular disease.

ApoE-deficiency is characterized by hypercholesterolemia, high oxidative stress, as well as increased inflammatory status and altered innate immune function. **Chapter 4** more specifically describes the role of maternal hypercholesterolemia in prenatal programming of cardiovascular disease risk by studying the effects of maternal Ldlr-deficiency on Ldlr^{+/-} and Ldlr^{-/-} offspring.

In **Chapter 5** we present data that adult vascular endothelial cells in vivo are capable of undergoing endothelial-to-mesenchymal-transformation. Through this transdifferentiation endothelial cells contribute to the total pool of mesenchymal cells that compose the neointima. The data indicate that endothelial-to-mesenchymal-transformation could be important in development and progression of cardiovascular disease.

Chapter 6 describes the role of a number of maternal signals that exert adverse effects on the intrauterine environment and subsequent cardiovascular disease risk in the offspring. Furthermore, we discuss several epigenetic mechanisms possibly underlying intrauterine programming leading to increased atherosclerosis susceptibility.

In **Chapter 7** the effect of maternal apoE-deficiency on DNA methylation and histone modifications in the vasculature of apoE^{+/-} offspring is investigated. We demonstrate that intrauterine exposure results in tissue-specific alterations in histone modification patterns in the vascular wall providing us with a first indication of an underlying epigenetic mechanism to explain the increased atherosclerosis susceptibility observed in these mice.

Chapter 8 provides a general discussion on the fetal origin of adult cardiovascular disease, the prenatal risk factors involved and possible underlying mechanisms. Moreover, the implications of these data on prevention, lifestyle management and treatment strategies for mothers, as well as offspring are pointed out.

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Chapter 2

Intrauterine Exposure to Maternal Atherosclerotic Risk Factors Increases the Susceptibility to Atherosclerosis in Adult Life

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Intrauterine Exposure to Maternal Atherosclerotic Risk Factors Increases the Susceptibility to Atherosclerosis in Adult Life

Abstract

Maternal hypercholesterolemia is associated with a higher incidence and faster progression of atherosclerotic lesions in neonatal offspring. We aimed to determine whether an in utero environment exposing a fetus to maternal hypercholesterolemia and associated risk factors can prime the murine vessel wall to accelerated development of cardiovascular disease in adult life. To investigate the epigenetic effect in utero, we generated genetically identical heterozygous apolipoprotein E-deficient progeny from mothers with a wild-type or apolipoprotein E-deficient background. A significant increase in loss of endothelial cell volume was observed in the carotid arteries of fetuses from apolipoprotein E-deficient mothers, but fatty streak formation was absent. Spontaneous atherosclerosis development was absent in the aorta and carotid arteries in adult life. We unilaterally placed a constrictive collar around the carotid artery to induce lesion formation. In offspring from apolipoprotein E-deficient mothers, collar placement resulted in severe neointima formation in 9 of 10 mice analyzed compared with only minor lesion volume (2 of 10) in the progeny of wild-type mothers. We conclude that the susceptibility to neointima formation of morphologically normal adult arteries is already imprinted during prenatal development and manifests itself in the presence of additional atherogenic risk factors in adult life. Future research will concentrate on the mechanisms involved in this priming process, as well as on prevention strategies.

Introduction

Postnatal risk factors of atherosclerosis are well-defined and mechanisms contributing to lesion formation are increasingly understood. Previous studies showed that the atherogenic process in humans can already start during fetal development.¹ In a morphometric postmortem analysis of atherosclerosis in fetuses and children (Fate of Early Lesions in Children Study),² it was demonstrated that specifically maternal hypercholesterolemia was associated with a higher incidence of atherosclerotic lesions during the fetal period and a faster progression of these atherosclerotic lesions after birth, even under conditions of normocholesterolemia in the offspring. A causal role for maternal hypercholesterolemia in fetal lesion formation was demonstrated in a genetically nonhomogeneous rabbit model of diet-induced hypercholesterolemia and confirmed in a low-density lipoprotein receptor-deficient mouse model (Ldlr^{-/-}).^{3,4} Raised cholesterol intake of pregnant Ldlr^{-/-} mice accelerated the progression of spontaneous atherosclerotic lesions in the Ldlr^{-/-} progeny. Because these mice have elevated plasma cholesterol levels independent of the maternal cholesterol levels during postnatal life, they are genetically prone to develop atherosclerotic lesions. The question remains whether solely environmental atherogenic risk factors present in utero can prime the developing vascular system.

We hypothesized that the offspring without a genetic predisposition for spontaneous atherosclerosis, which had been exposed in utero to maternal atherosclerotic risk factors, would show increased susceptibility for atherosclerosis development in adult life compared with offspring from wild-type mothers. To solely investigate the epigenetic effect, we generated genetically identical heterozygous apolipoprotein E-deficient mice (apoE^{+/-}) from mothers with a normocholesterolemic wild-type or a hypercholesterolemic apoE-deficient (apoE^{-/-}) background and studied the morphology of the carotid arteries during the fetal and adult period. Homozygous apoE-deficiency is associated with hypercholesterolemia and spontaneous development of extensive atherosclerosis resembling human lesion composition.^{5,6} ApoE^{+/-} mice on the other hand, only develop hypercholesterolemia and atherosclerosis when fed a high-fat Western-type diet.^{7,8} To study the effect of maternal atherosclerotic risk factors in utero, we examined the morphology of the vascular wall in the late fetal stages. To investigate a possible increased vulnerability to atherosclerosis in adulthood, we studied neointima formation in both groups after induction of lowered shear stress levels by perivascular constrictive collar placement around the carotid arteries.

Methods

Mice

The apoE^{-/-} mice used were generated by homologous recombination in embryonic stem cells as described and backcrossed ≥ 8 times to a C57Bl/6J background.⁸ Wild-type C57Bl/6J mice were purchased from Charles River Laboratories (Maastricht, The Netherlands, import agency for Jackson Laboratories). The apoE^{-/-} and wild-type mice were crossbred to generate genetically identical apoE^{+/-} fetuses (n=20 each group) from apoE^{-/-} mothers (n=5) as well as from wild-type mothers (n=5). To examine the long-term effects of prenatal exposure to risk factors for atherosclerosis, we used female apoE^{+/-} offspring (n=10 each group). After weaning (age 4 weeks), the animals received a Western-type diet (1% cholesterol, Hope Farms) for 16 weeks. Diet and water were provided ad libitum. The Committee on Animal Welfare, Leiden University Medical Center, approved all of the animal experiments.

Lipid Measurements

Total levels of cholesterol and triglycerides were enzymatically quantified in blood plasma of 4-hour fasted animals by using commercially available kits (Roche). Adult blood samples were obtained through tail bleeding. Maternal blood samples were acquired before pregnancy and at day 17.5 of pregnancy while fetal blood samples were acquired by decapitation at embryonic day 17.5 (E17.5). Samples of the offspring were acquired at the postnatal ages of 4, 8 and 16 weeks.

Carotid Collar placement

Neointima formation was induced in a random order by perivascular collar placement in apoE^{+/-} offspring and C57BL/6J controls (n=5) at the age of 16 weeks as previously described.⁹ Mice were anesthetized by intraperitoneal injection of Domitor (0.5 mg/kg, Pfizer), Dormicum (5 mg/kg, Roche) and Fentanyl (0.05 mg/kg, Janssen-Cilag). A constrictive silicone collar (0.31 mm ID, 0.64 mm OD, and 2.0 mm length, Helixmark) was placed around the left common carotid artery. After wound closing, anesthesia was antagonized with a subcutaneous injection of Antisedan (2.5 mg/kg, Pfizer), Anexate (0.5 mg/kg, Roche) and Naloxone (1.2 mg/kg).

Magnetic Resonance Microscopy

Visualization of the arterial tree was performed 4 weeks after collar placement. For detailed information on MRI see Appendix.

Tissue Harvesting and Preparation

At E17.5, fetal thorax tissue was removed and fixed in 4% paraformaldehyde in 0.1 mol/L of sodium phosphate buffer (pH 7.4) for 48 hours or embedded in TissueTek (Bayer) for nonfixated cryosections. Four weeks after collar placement, the mice were anesthetized and the thorax opened. Pressure-perfusion fixation (76 mm Hg) was performed through the cardiac left ventricle with PBS for 5 minutes followed by 4% paraformaldehyde in 0.1 mol/L of PHEM buffer (60 mmol/L of Pipes, 25 mmol/L of Hepes, 10 mmol/L of ethyleneglycoltetraacetic acid, and 2 mmol/L of $MgCl_2$; pH 6.97) for 5 minutes. The aorta and common carotid arteries were harvested and the collar was removed. Pressure-perfusion with only PBS was used for cryosections. The specimens were fixed for 48 and 6 hours, respectively, in 4% paraformaldehyde in 0.1 mol/L of PHEM buffer. After fixation, the tissue was dehydrated in ethanol and xylene and paraffin embedded. Transverse 5- μ m sections were cut and serially mounted.

Immunohistochemistry

Routine staining was performed with hematoxylin and eosin, Resorcin-Fuchsin for detection of elastin, Sirius red for collagen, and Oil Red O to assess lipid deposition. Unless indicated otherwise, the immunohistochemistry was performed as described.¹⁰ Sections were incubated overnight at room temperature with a mouse monoclonal primary antibody against α -smooth muscle actin to identify vascular smooth muscle cells (1:2000, Sigma Aldrich, Product No. A2547), a rat monoclonal anti-CD31 (1:50, PharMingen) and rabbit polyclonal anti-von Willebrand factor (vWF; 1:2000, DAKO) to study endothelial cells, and a rat monoclonal Mac-3 (1:400, PharMingen) against macrophages. Goat anti-rabbit biotin conjugate (1:200, Vector), goat anti-rat biotin conjugate (1:200, PharMingen), and rabbit anti-mouse peroxidase conjugate (1:200, DAKO) with normal goat and mouse serum diluted in PBS were used as secondary antibodies (1 hour at room temperature). Biotin labeling was followed by incubation with Vectastain ABC (Vector). The CD31 signal was enhanced with a colony-stimulating activity kit (DAKO). 3-3' Diaminobenzidine tetrahydrochloride was used as chromogen and counterstaining was performed with Mayer's hematoxylin.

Morphometry

To estimate the total volume of endothelial cells in the fetal carotid arteries at E17.5 the Cavalieri principle,¹¹ a point counting method using a grid, was used in 5 equally spaced sections stained with vWF (n=5 each group). In addition, morphometry was used to estimate the total volume of neointimal lesions in the

adult carotid artery. To exclude bias because of differences in maximum neointima area, the complete plaque was studied by volume measurements. Lumen and medial volume were also estimated. Contralateral noncompromised carotid arteries were assessed in a comparable region. Fifteen equally spaced sections were examined. The total intimal volume was measured between the endothelial cell monolayer and internal elastic lamina and total medial volume between the external and internal elastic lamina. The intima/media ratio was calculated by dividing the intimal volume by the medial volume.

Statistical Analysis

Data are represented as mean $\pm$ SEM. Paired and unpaired data were evaluated by 2-tailed Student's *t*-test. The differences were considered to be significant if $P < 0.05$.

Results

Lipid Metabolism

To study the effect of maternal hypercholesterolemia and associated risk factors on fetal lipid levels, we determined maternal and fetal plasma cholesterol and triglyceride levels. Maternal total plasma cholesterol and triglyceride levels are shown in Table 1. As expected, before and during pregnancy plasma cholesterol and triglyceride levels differed significantly between wild-type and apoE^{-/-} mothers. Plasma cholesterol concentrations in both groups significantly decreased during pregnancy. A striking reduction of 51% was observed in plasma cholesterol levels of apoE^{-/-} mothers at pregnancy day 17.5 but the cholesterol status remained hypercholesterolemic. Plasma triglyceride levels increased significantly in pregnant wild-type mothers, but not in the apoE^{-/-} mothers.

Maternal hypercholesterolemia in apoE^{-/-} mothers resulted in a significant plasma cholesterol elevation in apoE^{+/-} mice at E17.5 compared with fetuses from wild-type mothers (Table 2). The plasma cholesterol levels found in apoE^{+/-} offspring at weaning and in adult life after a Western-type diet were similar. Plasma triglyceride levels were low in all of the fetuses.

Table 1. Maternal plasma cholesterol and triglyceride levels

Mother	TC (mmol/L)	TG (mmol/L)
Wild-type		
Before pregnancy	1.98 ± 0.15	0.50 ± 0.03
Pregnancy day 17.5	1.33 ± 0.04*	1.58 ± 0.12*
ApoE^{-/-}		
Before pregnancy	20.13 ± 0.44 [‡]	1.50 ± 0.10 [‡]
Pregnancy day 17.5	9.42 ± 0.79* [‡]	2.08 ± 0.21 [‡]

All data are shown as mean ± SEM. TC indicates total plasma cholesterol;

TG, total plasma triglyceride. **P* < 0.05 versus before pregnancy,

[‡]*P* < 0.05 versus other group at same time point.

Fetal Carotid Artery Composition

To examine the effect of intrauterine exposure to maternal atherosclerotic risk factors on fetal vascular remodeling, we studied the morphology of the vascular wall of the carotid arteries in apoE^{+/-} fetuses from wild-type and apoE^{-/-} mothers at E17.5. No fatty streaks were observed in the carotid arteries of all fetuses. However, in carotid arteries of fetuses from apoE^{-/-} mothers increased endothelial cell damage was detected (Figure 1A). With light microscopic examination we

detected several areas in the carotid arteries where endothelial cells were absent. Morphometry revealed significantly less endothelial cell volume in the carotid arteries of apoE^{+/-} fetuses from apoE^{-/-} mothers compared with fetuses from wild-type mothers ($18.81 \pm 1.54 \times 10^4$ versus $24.81 \pm 1.11 \times 10^4 \mu\text{m}^3$; Figure 1B). We found no lipid deposition in the vascular wall. In addition, Mac-3 staining showed that macrophages were not present in the vascular wall (results not shown). Overall quantification of vWF staining intensity in individual endothelial cells showed no significant difference between the two groups.

Table 2. Offspring plasma cholesterol and triglyceride levels

Mother	Offspring	TC (mmol/L)	TG (mmol/L)
Wild-type	ApoE ^{+/-}		
	E17.5	1.54 ± 0.04	0.30 ± 0.01
	4 weeks	2.00 ± 0.03	0.93 ± 0.03
	8 weeks	6.21 ± 0.14	0.70 ± 0.02
	16 weeks	7.89 ± 0.31	0.44 ± 0.02
ApoE ^{-/-}	ApoE ^{+/-}		
	E17.5	$2.11 \pm 0.06^*$	0.37 ± 0.02
	4 weeks	1.90 ± 0.06	0.81 ± 0.05
	8 weeks	7.08 ± 0.21	0.56 ± 0.04
	16 weeks	7.73 ± 0.36	0.47 ± 0.02

All data are shown as mean $\pm$ SEM. TC indicates total plasma cholesterol; TG, total plasma triglyceride. * $P < 0.000$ at E17.5 versus other group at the same time point.

Carotid Artery Visualization by MRI

Four weeks after collar placement, the carotid arteries and the position of the collar could clearly be visualized with magnetic resonance microscopy in all of the C57BL/6J mice (Figure 2A). In 4 of 5 apoE^{+/-} mice born from wild-type mothers, the angiography revealed a normal carotid artery lumen proximal to the position of the collar (Figure 2B). In 1 case the left carotid artery could not be visualized as a result of thrombus formation within the collar (confirmed by histology). In the group of apoE^{+/-} mice from apoE^{-/-} mothers, only 1 angiography resembled the wild-type. In 2 cases the lumen of the left carotid artery was only partly visualized, whereas in the other 2 mice the left carotid artery lumen was completely absent (Figure 2C). Histology revealed that the lack of visualization was caused by severe lumen narrowing as a result of presence of intimal thickening proximal to the collar.

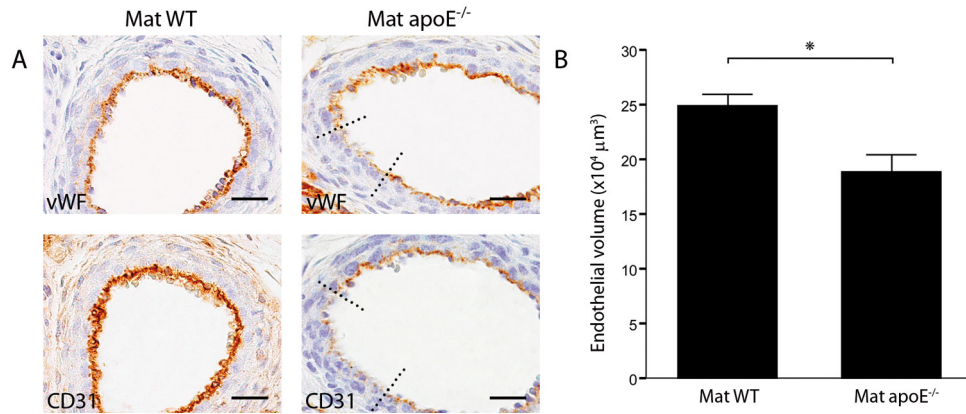


Figure 1. Effects of in utero exposure of apoE^{+/-} fetuses to maternal atherosclerotic risk factors on endothelial cells of the carotid arteries. (A) Representative immunostaining for vWF and CD31 in cross-sections from apoE^{+/-} fetuses from wild-type (Mat WT, left panels) and apoE^{-/-} mothers (Mat apoE^{-/-}, right panels) at age E17.5. Region between broken lines indicates an area of decreased expression of vWF and CD31, respectively. Scale bars, 20 μm. (B) Morphometric analysis of endothelial cell volume in the carotid arteries. Data are mean ± SEM (n=5 each). **P* < 0.01.

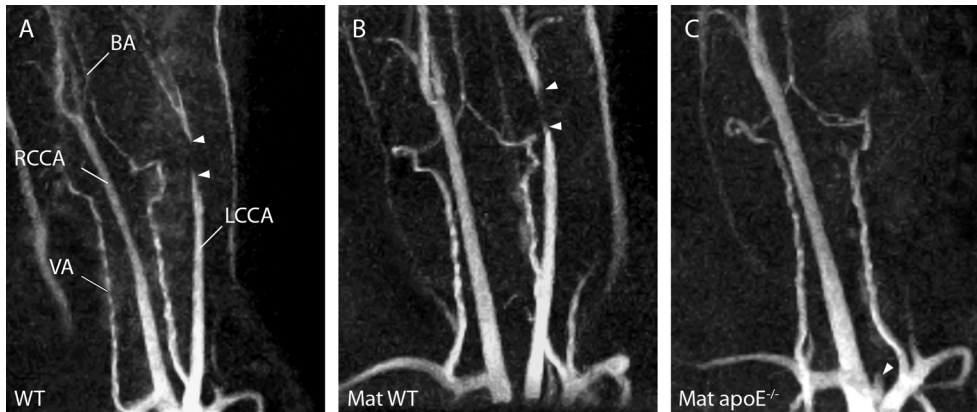


Figure 2. Effects of a constrictive collar four weeks after placement around the left common carotid artery. Angiographies representative for 20 weeks old (A) wild-type (WT), (B) apoE^{+/-} from wild-type mothers (Mat WT) and (C) apoE^{+/-} mice from apoE^{-/-} mothers (Mat apoE^{-/-}). The induced intimal thickening in Mat apoE^{-/-} reduces the flow to an undetectable level, only the trunk of the LCCA is visible. BA indicates basilar artery; VA, vertebral artery; RCCA, right common carotid artery; LCCA, left common carotid artery. Arrows indicate the region of the collar and in (C) the trunk of the LCCA.

Lesion Size

Spontaneous neointima formation was assessed in adult apoE^{+/-} offspring from wild-type and apoE^{-/-} mothers at the age of 20 weeks. No lesions were detected at the level of the aortic root and in the aortic arch (see Appendix). In addition, in noncompromised carotid arteries normal vascular wall morphology was observed (Figure 3A). We found that collar placement resulted in severe neointima formation proximal to the collar in apoE^{+/-} mice from apoE^{-/-} mothers (9 of 10) compared with minor lesions in the offspring from wild-type mothers (2 of 10) (Figure 3B-C). Significantly increased lesion volume was observed in offspring of apoE^{-/-} mothers ($5.04 \pm 1.65 \times 10^6$ versus $0.28 \pm 0.18 \times 10^6 \mu\text{m}^3$; $P = 0.018$; Figure 4A). A trend towards increased medial volume was observed as result of collar placement (Figure 4B). A significant increase in medial volume was detected between the noncompromised and the collared carotid arteries of in utero exposed apoE^{+/-} mice ($20.34 \pm 0.77 \times 10^6$ versus $29.96 \pm 2.56 \times 10^6 \mu\text{m}^3$; $P = 0.04$). Medial volumes between the contralateral arteries of both groups did not differ. The intima/media ratio was significantly increased in apoE^{+/-} mice from apoE^{-/-} mothers (0.15 ± 0.05 versus 0.01 ± 0.01 ; $P = 0.017$; Figure 4C). Severe lesions were characterized by a cylindrical structure and maximal intima/media ratios of ≈ 1.0 .

Lesion Composition

To assess the role of several cell lineages in intimal proliferation, neointimal lesion composition was studied. The endothelial cell lining was, in contrast to the findings in the fetuses, in all cases intact (Figure 5A). The vWF was absent in the endothelial cells and present subendothelially (Figure 5B). All lesions contained macrophages (Figure 5C). Extensive extracellular matrix deposition was demonstrated throughout the neointima by increased presence of elastin and collagen (Figure 5D-E). Enhanced medial volume in collared arteries appeared also to be the result of increased collagen production. All elastic lamina of the media were intact, thereby excluding major invasion of medial smooth muscle cells into the intima at this stage. Staining for α -smooth muscle actin was not restricted to the media, but positive cells were also detected in the intima (Figure 5F).

Oil red O staining revealed that Western-type diet alone did not result in lipid deposition in the carotid arteries (Figure 6A). A second trigger, collar placement, resulted in limited accumulation of lipids in the media only directly proximal to the collar in apoE^{+/-} mice from wild-type mothers (Figure 6B). On the contrary, massive lipid deposition was observed in the intima as well as in the media of collared arteries of apoE^{+/-} mice from apoE^{-/-} mothers (Figure 6C).

The vascular wall of all contralateral, noncompromised carotid arteries showed a normal morphology. In addition to immunohistochemical analysis of these arteries, we performed microarray analysis to detect changes in intrinsic gene expression patterns (see Appendix for preliminary data).

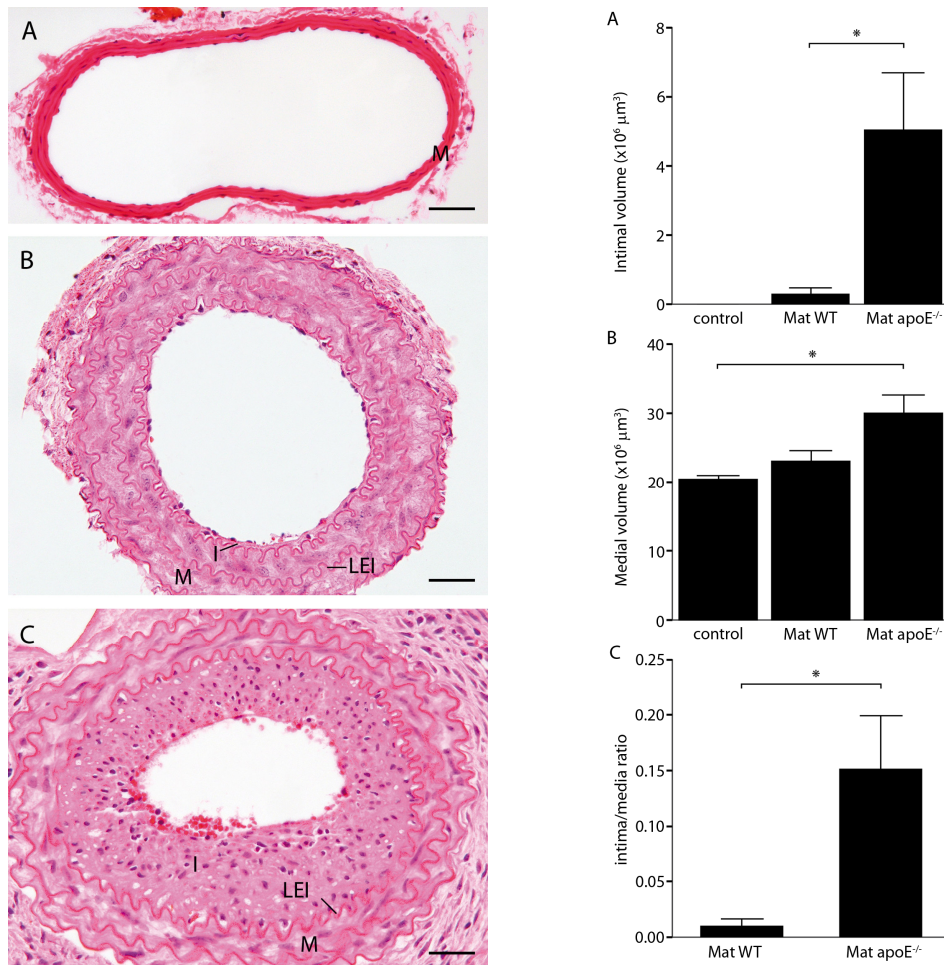


Figure 3. Morphology of adult carotid arteries. Mice were euthanized at 20 weeks of age, 4 weeks after collar placement. Perfusion-fixation induced dilatation of the arteries. Presence of intimal thickening prevented dilatation. Representative HE staining in cross-sections from (A) noncompromised carotid arteries and the region proximal to the collar in apoE^{-/-} mice from (B) wild-type and (C) apoE^{-/-} mothers, respectively. I indicates intima; M, media; LEI, lamina elastica interna. Scale bars: 30 μm.

Figure 4. Morphometric analysis of the effects of collar placement. (A) Estimation of intimal volume and (B) medial volume of noncompromised (control) and collared carotid arteries of apoE^{-/-} mice from wild-type (Mat WT) and apoE^{-/-} (Mat apoE^{-/-}) mothers. (C) Calculated intima/media ratio for the collared carotid arteries of both apoE^{-/-} groups. Data are mean ± SEM (n=10 each). *P < 0.05.

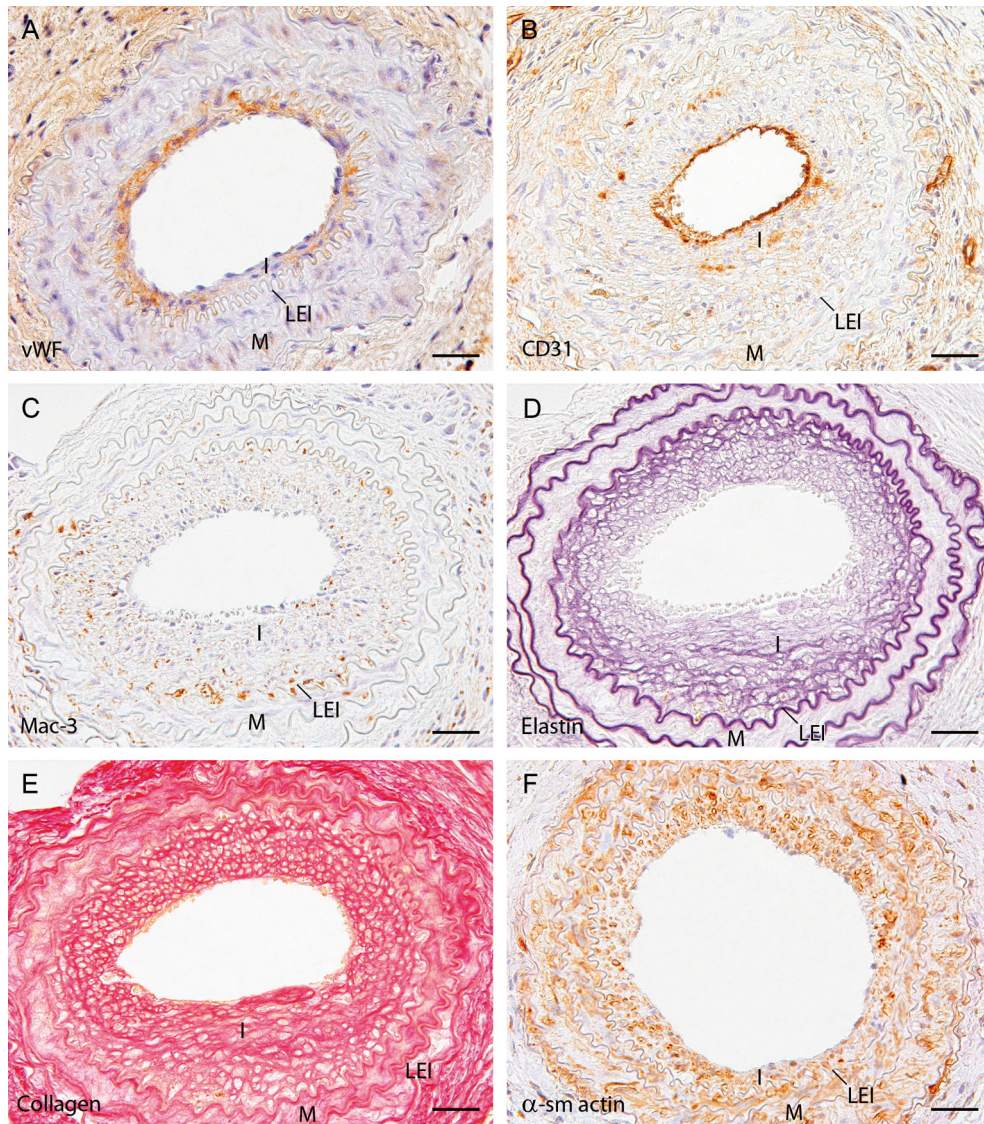
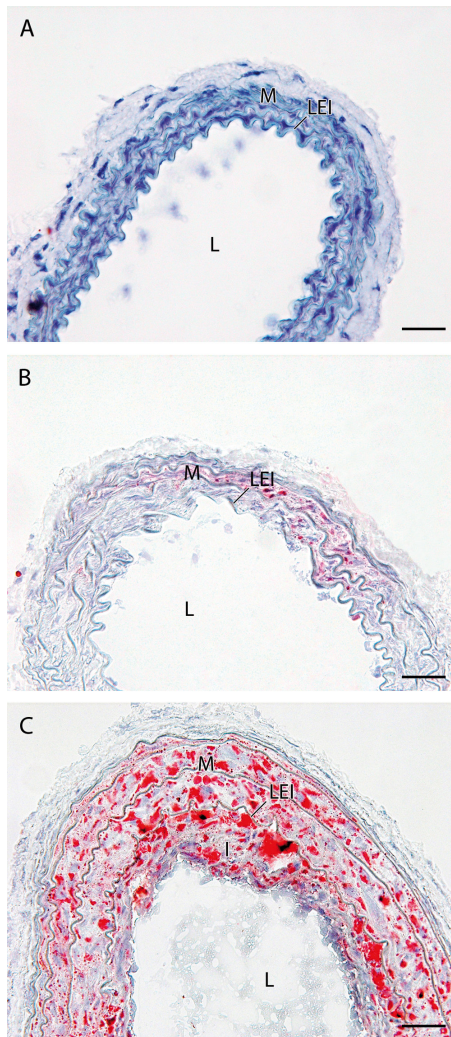


Figure 5. Neointima composition. Representative cross-sections from apoE^{+/+} mice from apoE^{-/-} mothers were stained to study (A and B) endothelial cells, (C) macrophages, (D and E) extracellular matrix production and (F) α-sm actin expression. I indicates intima; M, media; LEI, lamina elastica interna. Scale bars: 30 μm.

Figure 6. Oil Red O staining. Representative staining in cross-sections from (A) noncompromised carotid arteries and the region proximal to the collar in apoE^{+/+} mice from (B) wild-type and (C) apoE^{-/-} mothers. The collar induces deposition of lipids in the media in the low flow areas in B. Note the increase in lipid deposition in the Mat apoE^{-/-} offspring in C. I indicates intima; M, media; LEI, lamina elastica interna; L, lumen. Scale bars, 30 μm. ➡



Discussion

The present study has provided evidence that the susceptibility to atherosclerosis of morphologically normal adult vessels can already be imprinted during prenatal development. We showed that in utero exposure of apoE^{+/-} embryos and fetuses to environmental maternal atherosclerotic risk factors can prime the vasculature to severely aggravated neointima formation in morphological normal adult carotid arteries after collar placement compared with nonexposed offspring from wild-type mothers. In apoE^{+/-} mice the remaining apoE allele is sufficient to generate plasma apoE levels equal to those of wild-type animals (reviewed by Getz and Reardon¹²). Furthermore, plasma lipid levels of both groups of mice are indistinguishable when fed chow or a moderate Western-type diet,^{7,8} and spontaneous atherosclerosis development in the aortic root is absent. Homozygous apoE-deficiency in mice is associated with hypercholesterolemia,^{5,6} increased oxidative stress levels,¹³ and enhanced inflammatory status,¹⁴ and all of these factors may have a role in the process of

programming of the vessel wall of apoE^{+/-} offspring. However, contribution of other factors associated with apoE-deficiency cannot be excluded.

Because apoE-deficiency is associated with hypercholesterolemia, we assessed the effect of increased maternal cholesterol status on fetal cholesterol levels. In our analysis we found that maternal hypercholesterolemia enhanced fetal plasma cholesterol levels. Neonatal plasma cholesterol levels did not differ between apoE^{+/-} mice from apoE^{-/-} and wild-type mothers. Our data are in concordance with previous studies in humans, rats, and mice that demonstrated that no correlation exists between maternal and fetal plasma cholesterol levels late in pregnancy and at and after birth.^{1,15-17} However, we have no data available on fetal plasma cholesterol levels earlier in pregnancy. Because several reports

indicate that, during mid-pregnancy, fetal plasma cholesterol levels are very high,^{1,18} the slight significant difference observed at E17.5 may be a reflection of larger differences earlier in development.

Studies in human subjects revealed that fatty streaks are present in fetal aortas and carotid arteries, and the existence of maternal hypercholesterolemia throughout pregnancy leads to an increased number and size of the lesions.^{1,19} In human cerebral and coronary arteries on the other hand, no lipid accumulations and macrophages were detected prenatally.^{19,20} In rabbits, diet-induced maternal hypercholesterolemia results in extensive lesion formation in the aorta of the offspring at birth.³ Our data show that the presence of maternal atherosclerotic risk factors leads to endothelial cell activation and damage in carotid arteries of apoE^{+/-} fetuses. Except for these early markers of atherosclerosis,²¹ no morphological abnormalities, e.g. fatty streaks, were observed in the murine fetal carotid arteries.

Furthermore, no intimal thickening was detected in the aorta of the fetuses (unpublished data). Anatomical and functional differences in arteries, as well as discrepancies between species, may explain the observed distinction in atherosclerotic lesion formation in fetal life. Because apoE^{+/-} mice from apoE^{-/-} mothers lack atherosclerotic lesions in fetal vasculature and spontaneous lesion formation in the aortic root, aortic arch, and carotid arteries at the age of 20 weeks, our model is very useful to study the long-term consequences of imprinting of morphologically normal vessels.

ApoE-deficiency leads to high levels of reactive oxygen species in the circulation and tissues.²² In addition, it has been described that atherosclerosis development in these mice is accompanied by a reduction in antioxidant enzymes in the athero-prone areas.²³ It has been suggested that reactive oxygen species pass the placenta²⁴ and as a result may influence the oxidative status of the fetus. Napoli and colleagues³ and Palinski and colleagues²⁵ reported that addition of vitamin E or cholestyramine to the diet of rabbits significantly diminished atherosclerosis in the offspring. Oxidative stress has also been described to affect DNA methylation.^{26,27} As a consequence, embryonic development, as well as other physiological processes may be modulated by increased oxidative stress. We hypothesize that maternal hypercholesterolemia through oxidative stress affects signaling processes in the cell lineages of the fetal vascular wall and bone marrow leading to functional changes in these cells. That epigenetic changes are likely to be responsible for the aggravated atherogenic response to collar-induced shear stress changes in adult life is currently a focus of our ongoing investigations.

There seems to be a paradox between our study and a previous study by Madsen and colleagues¹⁵ who did not observe an effect of in utero exposure to maternal atherosclerotic risk factors on atherosclerotic lesion formation in adult life.

In the latter study, spontaneous lesion formation was induced by a Western-type diet containing the strong atherosclerosis-inducing factor cholate. In long-term survivors, they detected equal degrees of advanced atherosclerosis. In our study, spontaneous expression of atherosclerosis was missing, and we studied the initial stages of neointima formation, which turned out to be different between the two groups of apoE^{+/-} mice. Therefore, our studies cannot be reliably compared.

The striking differences in adult lesion formation between the in utero exposed and nonexposed apoE^{+/-} mice raise the question which cell types involved are affected during embryonic development. The most obvious vascular cell populations to be considered are the endothelial and smooth muscle cells of the carotid arteries and the circulating bone marrow-derived cells. Changes in the gene expression profiles of mural cells after in utero exposure to high cholesterol levels has been recently demonstrated in Ldlr^{-/-} mice.⁴ Diet-induced maternal hypercholesterolemia in Ldlr^{-/-} mothers significantly altered the expression of 139 genes in descending aortas of Ldlr^{-/-} offspring without signs of lesion formation. These data are in line with our gene set enrichment analysis of the microarray data of noncompromised carotid arteries showing changes in gene expression as a result of in utero exposure to maternal atherosclerotic risk factors (see Appendix). This method allows insight into the dynamics of genes contributing to a gene set without having to work within the fixed thresholds that genes have to meet to be differentially expressed and demonstrated altered regulations of various pathways in the vascular wall. Larger sample sizes of carotid arteries and descending aortas in combination with laser dissection microscopy will give us insight into the vulnerability of the individual cell populations for induction of changes in gene expression as a consequence of prenatal exposure to atherosclerotic risk factors.

In conclusion, the present study has provided evidence that the susceptibility to atherosclerosis of morphologically normal adult vessels is already imprinted during prenatal development and is not genetically determined in our model. In the presence of additional risk factors, neointima formation in adult life is severely accelerated. Our findings indicate that the basis for development of atherosclerosis in adult life is already laid down during embryonic development. Risk factors for atherosclerosis in human adults are mainly associated with lifestyle. Extensive lifestyle management can, thus, reduce the number of environmental triggers that induce aggravated atherosclerotic lesion formation. Future research will concentrate on the mechanisms involved in the priming process, as well as on prevention strategies.

Acknowledgements

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Appendix

Methods

Magnetic Resonance Microscopy

Mice were imaged in vivo using a vertical 9.4T (400 MHz), 89 mm bore magnet with a shielded 1T/m gradient and a transmit/receive birdcage radiofrequency coil with an inner diameter of 30 mm (Bruker Biospin, Rheinstetten, Germany). Bruker ParaVision 3.02 software was used for image acquisition and analysis. Mice (n=5 each group) were initially anesthetized with 4% isoflurane (Abbott, Hoofddorp, The Netherlands) in air (0.3 l min⁻¹) and O₂ (0.3 l min⁻¹) and maintained with ~1.5% isoflurane during all procedures. Anesthetized mice were mounted into a probehead and inserted head-up into the magnet. The respiratory rate was monitored via an air-pressure cushion connected to a laptop using Biotrig software (Bruker, Rheinstetten, Germany). The depth of the anesthesia was continuously regulated to maintain a stable respiration rate during the experiment. We used a 3D FLASH (fast low angle shot) sequence with an echo time of 1.85 ms, a repetition time of 30 ms, a 0.8 ms sinc radio frequency pulse with a flip angle of 30 degrees and a spectral band width of 100 kHz. With a field of view (FOV) of 20x20x20 mm, a matrix of 256x256x256 an isotropic resolution of 78 µm was reached within a scan time of 32 minutes and 46 seconds. 3D angiograms were generated in ParaVision by maximum intensity projection.

Microarray

Total RNA was isolated from noncompromised contralateral carotid arteries at the age of 20 weeks of 3 apoE^{+/-} offspring from wild-type mothers and 4 offspring from apoE^{-/-} mothers. RNA was isolated with the RNAqueous[®] Micro Kit (Ambion, Cambridgeshire, UK) according to the manufacturers protocol. The RNA concentration was determined by absorbency at 260 nm with a NanoDrop (Isogen Life Science B.V., IJsselstein, The Netherlands) and RNA integrity was verified by use of the RNA 6000 Pico assay on a Agilent 2100 Bioanalyzer (Agilent Technologies, Amstelveen, The Netherlands). All of the RNA was subjected to a first round of oligo-dT primed amplification with the MessageAmpTMII aRNA Amplification Kit (Ambion). One µg of aRNA was used in a random primed second round of amplification (MessageAmpTMII-Biotin Enhanced Kit, Ambion) to obtain biotin labeled aRNA. Hybridization, washing and scanning of mouse genome 430A GeneChip arrays (Affymetrix) was performed according to Affymetrix standard

protocols (<http://www.affymetrix.com>). After scanning, the images were quantified using the Affymetrix GeneChip Operating software. The scaled data were then imported into the R software environment (<http://www.R-project.org>) and analyzed with the Bioconductor (<http://www.bioconductor.org>) R/affy package. Normalization was performed by the Robust Multi-array Average method.¹ Gene expression levels were analyzed with the R/limma package (<http://www.bioconductor.org>) and corrected for multiple testing by false discovery rate (FDR) according to Benjamini and Hochberg.²

Results

Spontaneous Atherosclerosis

ApoE^{+/-} mice only develop atherosclerosis when fed a high fat Western-type diet. To examine the extent of spontaneous lesion development in our mouse model, the aortas of the apoE^{+/-} mice from apoE^{-/-} and wild-type mothers were examined at the termination of the experiment, at the age of 20 weeks. No atherosclerotic lesions were observed at the level of the aortic root (Figure 1A). Furthermore, no lesions were observed in the aortic arch and at the bifurcations (Figure 1B).

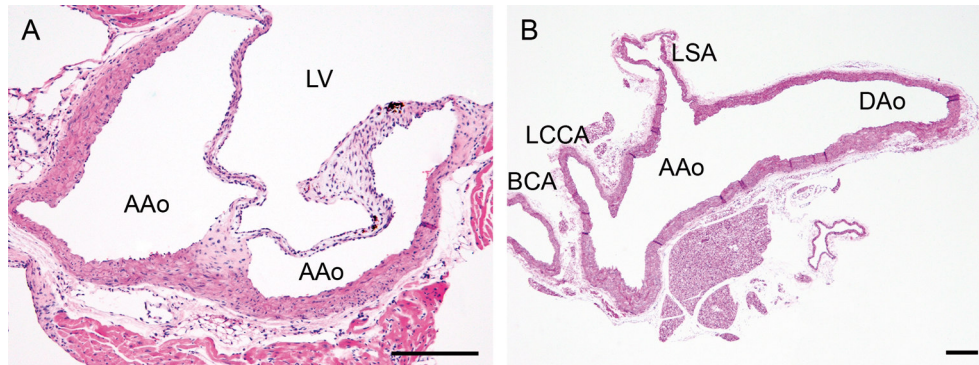


Figure 1. Analysis of spontaneous atherosclerosis. Pictures representative for (A) aortic root and (B) aortic arch. No lesions detectable. AAO indicates ascending aorta; LV, left ventricle; BCA, brachiocephalic artery; LCCA, left common carotid artery; LSA, left subclavian artery; AoA, aortic arch; DAO, dorsal aorta. Scale bars: (A) 200 μ m and (B) 300 μ m.

Gene Expression

Given the effect of intrauterine exposure to atherosclerotic risk factors on fetal carotid arteries and atherosclerotic lesion formation in adult life, we performed microarray analysis on the noncompromised carotid arteries of the apoE^{+/-} mice from both wild-type and apoE^{-/-} mothers to detect changes in intrinsic gene expression patterns. Up to 3309 genes had an unadjusted *P*-value of < 0.05 and 626 an unadjusted *P*-value of < 0.01. After correction for multiple testing no differentially expressed genes were detected. To exclude the possibility that relevant biological differences were not detected due to the relative noise inherent to the microarray technology, a gene set enrichment analysis was performed.³ This analysis revealed a number of upregulated pathways among which the CTLA-4 and TCRA T-cell activating pathway, the pro-inflammatory IL-12/STAT-4 pathway as well as pathways involved in fatty acid and the carbohydrate metabolism

(Table 1). The complete microarray analysis is available at <http://www.ncbi.nlm.nih.gov/geo/>, query GSE6134. Although the amount of samples available for the microarray analysis was limited, the nominal P -value was significant for the top ranked pathways. Therefore, the results may give rise to hypotheses for future research.

Table 1. Top ranked pathways gene set enrichment analysis.

ID	Pathway	nominal P -value
1	TCA	0.000
2	MAP00190_Oxidative_phosphorylation	0.000
3	FA	0.000
4	Krebs-TCA_Cycle	0.000
5	GO_0005739	0.000
6	TcytotoxicPathway	0.037
7	CR_immune_function	0.038
8	TcraPathway	0.040
9	Ctla4Pathway	0.040
10	Il17Pathway	0.042
11	Tob1Pathway	0.043
12	IL12Pathway	0.047
13	No2il12Pathway	0.049

Nominal P -value = P -value without correction for multiple testing.

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Chapter 3

Maternal ApoE-Deficiency Promotes Collar-Induced Neointima Formation in Adult ApoE^{+/-} Offspring Even Without Cholesterol Feeding

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Abstract

We have previously shown that adult apoE^{+/-} offspring from apoE^{-/-} mothers exhibit increased susceptibility to cardiovascular disease, as compared to adult apoE^{+/-} offspring from wild-type mothers. In this model, neointimal lesions, as a reflection of cardiovascular disease, were induced by placement of a constrictive collar around the carotid artery in combination with a high-cholesterol diet. The aim of the present study was to investigate whether adult apoE^{+/-} offspring from apoE^{-/-} mothers are also more susceptible to neointima formation without feeding a cholesterol-containing diet. On a chow diet, both types of apoE^{+/-} offspring were normocholesterolemic. After placement of a constrictive collar, lesions could not be detected in apoE^{+/-} offspring from wild-type mothers, whereas apoE^{+/-} offspring from apoE^{-/-} did develop neointimal lesions. These neointimal lesions were small and relatively a-cellular. The mesenchymal cells stained positive for α -smooth muscle actin and were negative for the macrophage marker Mac-3. We conclude that intrauterine exposure to maternal apoE-deficiency is sufficient to allow collar-induced neointima formation, even without cholesterol feeding, sustaining the hypothesis that the intrauterine environment poses a major risk factor for adult cardiovascular disease.

Introduction

Although the exact cause of atherosclerosis is unknown, many risk factors have been identified, for example age, gender, smoking, hypertension, hypercholesterolemia, obesity, and lack of physical exercise. These factors accumulate throughout life and operate synergistically, thereby promoting earlier and more severe onset of disease.

In addition to adult onset risk factors for atherosclerosis, prenatal risk factors have been identified. The “fetal origins hypothesis” postulated by Barker¹ proposes that adjustment to an unfavorable maternal environment is beneficial to the developing embryo. However, these adaptations may lead to an increased disease risk as the adult environment differs from the fetal situation. Examples of prenatal risks are fetal undernutrition and maternal hypercholesterolemia. Fetal undernutrition leads to persisting changes in a range of metabolic processes in the fetus thereby enhancing cardiovascular disease risk in adulthood.²⁻⁴ Intrauterine exposure to maternal hypercholesterolemia on the other hand, has been reported to enhance fatty streak formation, as well as lesion size in aortas of fetuses from hypercholesterolemic mothers compared with fetuses from normocholesterolemic mothers.⁵ After birth, lesion progression remained accelerated in these children in relation to children from normocholesterolemic mothers.⁶

To study the long-term consequences of intrauterine exposure to environmental maternal atherosclerotic risk factors such as hypercholesterolemia, we have used a genetically homogeneous mouse model. We were able to demonstrate increased endothelial cell activation and vascular injury in heterozygous apolipoprotein E-deficient ($\text{apoE}^{+/-}$) fetuses from homozygous apolipoprotein E-deficient ($\text{apoE}^{-/-}$) mothers as compared to genetically identical $\text{apoE}^{+/-}$ fetuses from wild-type mothers.⁷ Combined constrictive collar placement around the left common carotid artery and high-cholesterol feeding in adult $\text{apoE}^{+/-}$ offspring severely aggravated neointima formation in $\text{apoE}^{+/-}$ offspring from $\text{apoE}^{-/-}$ mothers whereas almost no lesions were found in $\text{apoE}^{+/-}$ offspring from wild-type mothers. However, it remained unclear whether solely intrauterine exposure to maternal apoE-deficiency was sufficient for increased development of cardiovascular disease in adult life.

Therefore, in this study we investigated whether adult $\text{apoE}^{+/-}$ offspring from $\text{apoE}^{-/-}$ mothers are also more susceptible for neointima formation in absence of a high-cholesterol diet. We generated genetically identical $\text{apoE}^{+/-}$ offspring from normocholesterolemic wild-type mothers, as well as from hypercholesterolemic $\text{apoE}^{-/-}$ mothers. Mice heterozygous for the apoE allele are comparable to wild-type mice regarding their lipid levels and their relative resistance to atherosclerosis.⁸⁻¹⁰

Even when fed a diet containing cholesterol, these mice do not develop atherosclerosis spontaneously up to the age of 20 weeks.⁷ The probability that apoE^{+/-} mice will develop atherosclerosis spontaneously during their life time is quite low. To investigate the consequences of prenatal priming on the susceptibility for cardiovascular disease, neointimal lesions, as a reflection of cardiovascular disease, were induced by placement of a constrictive collar around the left common artery of both apoE^{+/-} offspring from wild-type mothers and apoE^{+/-} offspring from apoE^{-/-} mothers. With the collar technique we can mimic the normal aging process and at the same time accelerate the process of atherosclerosis. The high-cholesterol diet that was used in our previous study⁷ was replaced by regular chow to maintain normocholesterolemic conditions throughout the experiment.

Methods

Animals

The apoE^{-/-} and wild-type C57Bl/6J mice were purchased from Charles River Laboratories (Maastricht, The Netherlands, import agency for Jackson Laboratories). ApoE^{-/-} mothers were crossed with wild-type fathers and vice versa to generate genetically identical apoE^{+/-} offspring from wild-type as well as from apoE^{-/-} mothers. Female offspring (n=5 each group) were used for experiments. Regular chow and water were provided ad libitum. The Committee on Animal Welfare, Leiden University Medical Center, approved all animal experiments.

Lipid Measurements

Total levels of cholesterol and triglycerides were enzymatically quantified in blood plasma of 4 hour fasted offspring at age 4, 8 and 16 weeks by using commercially available kits (Roche, Almere, The Netherlands). Blood samples were obtained through tail bleeding.

Neointimal Lesion Induction

Neointima formation was induced by placement of a constrictive collar around the left common carotid artery in all apoE^{+/-} animals at the age of 16 weeks, as previously described.^{7,11}

Tissue Harvesting and Preparation

Mice were anesthetized 4 weeks after collar placement. Pressure-perfusion (76 mm Hg) was performed through the cardiac left ventricle with sterile PBS for 5 minutes. Both common carotid arteries were harvested and the collar was removed. The carotid arteries were fixed in 4% paraformaldehyde in 0.1M sodium phosphate buffer (pH 7.4) for 6 hours. Fixation was followed by dehydration in series of ethanol and xylene and tissues were paraffin-embedded. Transverse 5- μ m sections were cut and serially mounted.

Immunohistochemistry

Routine staining was performed with Mayer's hematoxylin and eosin, Resorcin-Fuchsin for detection of elastin and Sirius red for collagen. Unless indicated otherwise the immunohistochemistry was performed as described earlier.^{7,12} In short, for each staining all sections were stained in one batch. The sections were incubated overnight at room temperature with a mouse monoclonal primary antibody against α -smooth muscle actin to identify vascular smooth muscle cells

(1:2000, Sigma Aldrich, Product No. A2547, Zwijndrecht, The Netherlands), a rabbit polyclonal anti-von Willebrand factor (vWF; 1:2000, DAKO, Glostrup, Denmark) and a rat monoclonal anti-CD31 (1:50, PharMingen, Alphen, The Netherlands) to study endothelial cells, and a rat monoclonal Mac-3 (1:400, PharMingen, Alphen, The Netherlands) against macrophages. Goat anti-rabbit biotin conjugate (1:200, Vector, Amsterdam, The Netherlands), goat anti-rat biotin conjugate (1:200, PharMingen) and rabbit anti-mouse peroxidase conjugate (1:200, DAKO) with normal goat and mouse serum diluted in PBS were used as secondary antibodies (1 hour at room temperature). Biotin labeling was followed by incubation with Vectastain ABC (Vector). The CD31 signal was enhanced with a CSA kit (DAKO). The 3-3' diaminobenzidine tetrahydrochloride was used as chromogen and counterstaining was performed with Mayer's hematoxylin.

Morphometry

The Cavalieri principle¹³ was used to estimate the volume of the neointimal lesions in the region proximal to the collar. The lesion area was counted in 20 equally spaced sections and the volume calculated. Medial volumes were estimated in 10 equally spaced sections comprising a total length of 350 μm proximal to the collar. As a control, intimal and medial volumes were calculated in contralateral noncompromised carotid arteries.

Statistical Analysis

Data are represented as mean $\pm$ SEM. The data on offspring weight and plasma lipid levels were evaluated by general linear model repeated measures. The Fisher's Exact test and ANOVA were used to statistically substantiate the results on neointima formation. The differences were considered to be significant if $P < 0.05$ and the power $\geq 80\%$.

Results

Body Weight and Lipid Levels

Throughout the experiment, no significant differences were observed between the body weights of apoE^{+/-} offspring from apoE^{-/-} mothers and apoE^{+/-} offspring from wild-type mothers (Table 1). Intrauterine exposure to maternal apoE-deficiency did not affect the total plasma cholesterol (Table 2) and triglyceride levels (Table 3) of the offspring.

Table 1. Weight offspring

Offspring	Weight (g)		
	4 weeks	8 weeks	16 weeks
Mat WT	10.3 ± 1.2	19.8 ± 0.6	23.3 ± 0.6
Mat apoE ^{-/-}	14.5 ± 1.0*	19.9 ± 0.3	24.1 ± 0.2

All data are shown as mean ± SEM. Mat WT indicates apoE^{+/-} offspring from wild-type mothers; Mat apoE^{-/-}, apoE^{+/-} offspring from apoE^{-/-} mothers. **P* = 0.027 versus Mat WT at 4 weeks.

Table 2. Total plasma cholesterol levels offspring

Offspring	TC (mmol/L)		
	4 weeks	8 weeks	16 weeks
Mat WT	1.30 ± 0.11	1.84 ± 0.20	1.74 ± 0.17
Mat apoE ^{-/-}	1.54 ± 0.10	1.74 ± 0.12	1.69 ± 0.08

All data are shown as mean ± SEM. TC indicates total plasma cholesterol; TG, total plasma triglycerides; Mat WT, apoE^{+/-} offspring from wild-type mothers; Mat apoE^{-/-}, apoE^{+/-} offspring from apoE^{-/-} mothers. No significant differences were found between the two groups at all time points.

Table 3. Total plasma triglyceride levels offspring

Offspring	TG (mmol/L)		
	4 weeks	8 weeks	16 weeks
Mat WT	0.52 ± 0.08	0.75 ± 0.06	0.55 ± 0.04
Mat apoE ^{-/-}	0.62 ± 0.04	0.68 ± 0.08	0.68 ± 0.05

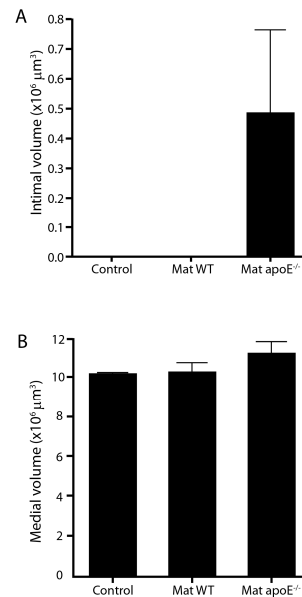
All data are shown as mean ± SEM. TC indicates total plasma cholesterol; TG, total plasma triglycerides; Mat WT, apoE^{+/-} offspring from wild-type mothers; Mat apoE^{-/-}, apoE^{+/-} offspring from apoE^{-/-} mothers. No significant differences were found between the two groups at all time points.

In contrast to our previous study in which the apoE^{+/-} offspring were fed a high-cholesterol diet to induce hypercholesterolemia,⁷ all mice received regular chow to maintain normocholesterolemia. In this way, the effects of intrauterine exposure to

maternal apoE-deficiency alone on cardiovascular disease risk could be examined. With age, a slight increase in total plasma cholesterol was observed in all animals, but levels remained within normal range.

Neointima Formation

The long-term consequences of intrauterine exposure to maternal apoE-deficiency were determined by studying the response of the vascular wall of both apoE^{+/-} offspring from wild-type mothers and those from apoE^{-/-} mothers after collar placement. Four weeks after collar placement, a significantly different response was observed between the two groups of mice. In 4 out of 5 apoE^{+/-} offspring from apoE^{-/-} mothers neointimal lesions were detected whereas no neointimal lesions were found in any apoE^{+/-} offspring from wild-type mothers (4 out of 5 versus 0 out of 5; $P = 0.024$). The average lesion volume in the apoE^{-/-} mice from apoE^{-/-} mothers was $0.49 \pm 0.28 \times 10^6 \mu\text{m}^3$ (Figure 1A). In addition, a trend towards an increased medial volume in the proximal region of the collar was observed in apoE^{+/-} offspring from apoE^{-/-} mothers ($10.33 \pm 0.47 \times 10^6 \mu\text{m}^3$) compared with those from wild-type mothers ($9.43 \pm 0.42 \times 10^6 \mu\text{m}^3$) and contralateral noncompromised carotid arteries ($9.37 \pm 0.02 \times 10^6 \mu\text{m}^3$, Figure 1B).



Carotid Artery Morphology and Lesion Composition

To characterize the morphology of the adult carotid arteries and the neointimal lesions, several histological and immunohistochemical staining techniques were used. In apoE^{+/-} offspring from wild-type mothers, the region proximal to the collar resembled the normal morphology as observed in noncompromised contralateral arteries (Figure 2A-B, D-E and G-H). In response to collar placement, neointimal lesions of 1 to 2 subendothelial layers developed in apoE^{+/-} progeny from apoE^{-/-} mothers (Figure 2C). One lesion per carotid artery was detected. The lesions were relatively a-cellular. Extensive deposition of the extracellular matrix proteins elastin and collagen was demonstrated (Figure 2F and 2I). Furthermore, thickening of the media and disorganization of the smooth muscle cells were detected in comparison to apoE^{+/-} offspring from wild-type mothers (Figure 2C,F and I). The internal elastic lamina was intact indicating the immaturity of the lesions (Figure 2F).

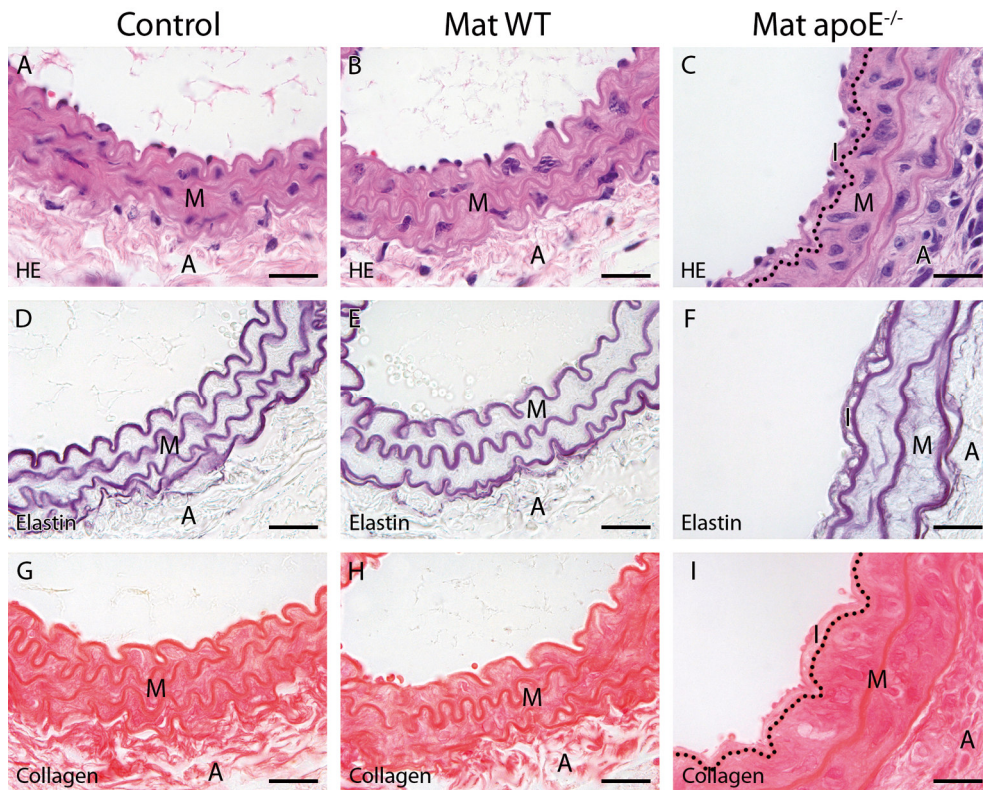


Figure 2. Histological staining. Representative cross-sections from (A,D,G) noncompromised carotid arteries and the region proximal to the collar in apoE^{-/-} mice from (B,E,H) wild-type mothers and (C,F,I) apoE^{-/-} mothers, respectively. Sections were stained with Mayer's (A-C) hematoxylin and eosine, (D-F) Resorcin-Fuchsin and (G-I) Sirius red. The dotted line represents the border between intima and media. I indicates intima; M, media; A, adventitia. Scale bars: 20 μ m.

◆ **Figure 1.** Morphometric analysis of adult carotid arteries 4 weeks after collar placement. (A) Estimation of intimal volume and (B) medial volume of noncompromised (control) and collared carotid arteries of apoE^{-/-} mice from wild-type mothers (Mat WT) and apoE^{-/-} mothers (Mat apoE^{-/-}). Data are means $\pm$ SEM (n = 5 each).

The CD31 staining revealed an intact endothelial lining in all carotid arteries (Figure 3A-C). The mesenchymal cells in the neointima were negative for CD31. The endothelial cells showed an activated phenotype. In comparison to control arteries in which all endothelial cells stained positively for anti-vWF, increased activation was observed in apoE^{+/-} from wild-type mothers (Figure 3D-E). In apoE^{-/-} mice from apoE^{-/-} mothers increased turnover and upregulation of vWF was seen in endothelial cells in the region proximal to the collar resulting in intense staining for vWF in the neointima (Figure 3F). In addition, vWF was detected in the media and

adventitia in these animals. Besides α -smooth muscle actin staining in the media, as also seen in the noncompromised contralateral arteries en those of apoE^{+/-} offspring from wild-type mothers, additional positivity was observed in the neointima of apoE^{+/-} mice from apoE^{-/-} mothers (Figure 3G-I). Macrophages were present abundantly in the adventitia and only sporadic presence was seen in the neointima and media of apoE^{+/-} mice from apoE^{-/-} mothers, (Figure 3L). The noncompromised control arteries and those of apoE^{+/-} offspring from wild-type mothers were completely devoid of macrophages (Figure 3J-K).

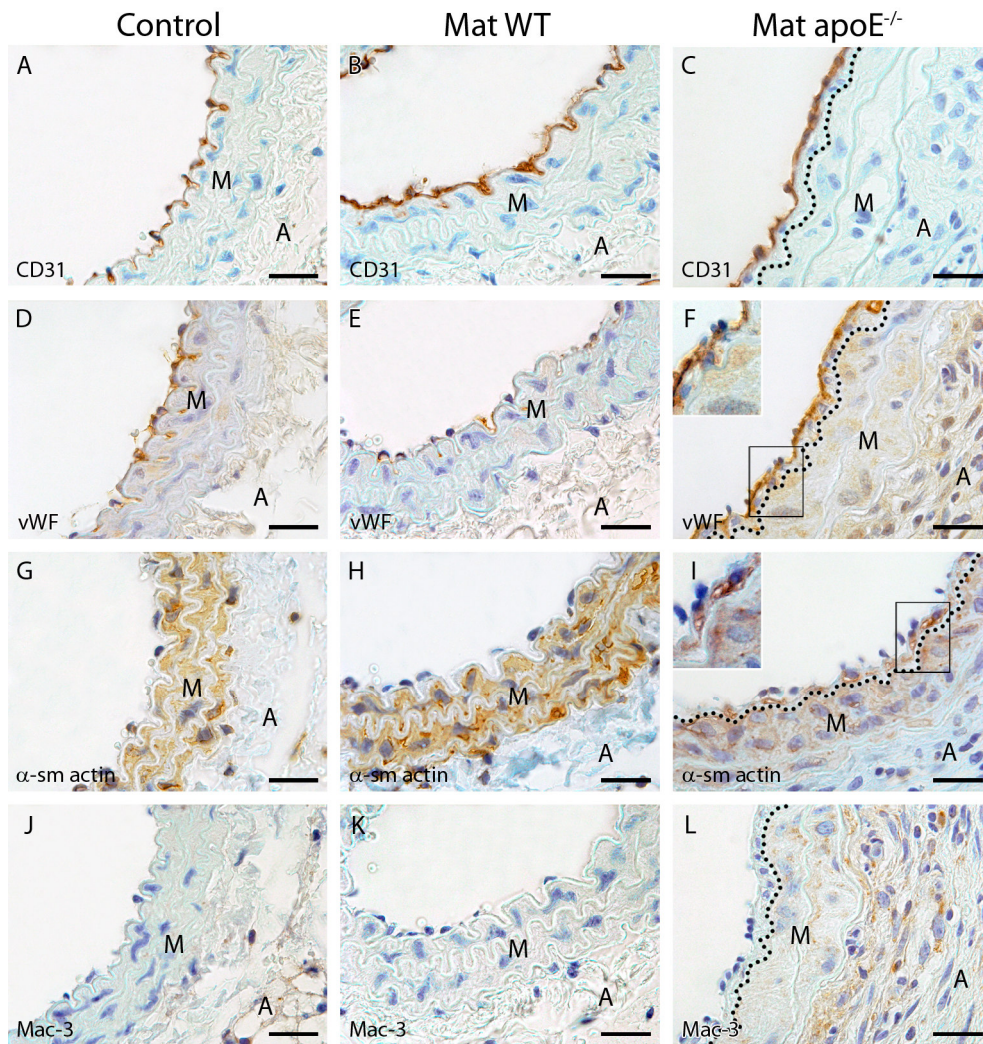


Figure 3. Immunohistological characterization of the vascular wall. Representative cross-sections from (A,D,G,J) noncompromised carotid arteries and the region proximal to the collar in apoE^{-/-} mice from (B,E,H,K) wild-type mothers and (C,F,I,L) apoE^{-/-} mothers, respectively. Sections were stained with (A-C) anti-CD31, (D-F) anti-vWF, (G-I) α-smooth muscle actin (α-sm actin) and (J-L) anti-Mac-3 to identify macrophages. Note the presence of vWF and α-sm actin in the neointima. The dotted line represents the border between intima and media. I indicates intima; M, media; A, adventitia. Scalebars: 20 μm.

Discussion

The present results demonstrate that high-cholesterol feeding is not required in the process of initiation of neointimal lesion development in apoE^{+/-} offspring from apoE^{-/-} mothers. Obviously, intrauterine exposure to maternal apoE-deficiency has a dramatic effect on atherosclerosis development in adult life. A shift from athero-protective high shear stress levels towards athero-prone low shear stress profiles within the carotid arteries as a result of collar placement was already sufficient to induce neointimal lesion formation in chow-fed normocholesterolemic apoE^{+/-} offspring from apoE^{-/-} mothers. No lesions were observed in apoE^{+/-} mice from wild-type mothers. These data indicate that the intrauterine environment poses a major risk factor for adult cardiovascular disease and can exert its effects independent of hypercholesterolemia induced by a cholesterol-containing diet.

The neointimal lesions in the carotid arteries of apoE^{+/-} offspring from apoE^{-/-} mothers were of substantial size and could be characterized by 1 or 2 subendothelial layers and relative a-cellularity. Whereas large numbers of macrophages were recruited into the adventitia, only sporadic presence of macrophages was seen in the neointima of apoE^{+/-} offspring from apoE^{-/-} mothers. In our previous study we used an identical approach as in the current study, except for the additional use of a cholesterol-containing diet.⁷ In the presence of diet-induced hypercholesterolemia large lesions developed and massive accumulation of macrophages was observed in apoE^{+/-} offspring from apoE^{-/-} mothers. Together, these data indicate that high-cholesterol feeding accelerates and aggravates neointimal lesion progression. Lesion progression seems to be accelerated through increased recruitment of inflammatory cells to the vascular wall. It has been reported that rolling of monocytes over the endothelial layer is increased in mice fed a cholesterol-containing diet.¹⁴ Moreover, adhesion molecules like VCAM-1 and P-selectin, are enhanced under these conditions.^{15,16} The lack of macrophages in the lesions observed in the current study could be the result of limited active recruitment of inflammatory cells from the circulation. Future studies have to reveal whether the current immature lesions observed in the 5 months old chow-fed apoE^{+/-} offspring from apoE^{-/-} mothers will acquire a large numbers of macrophages, associated fat deposition, and remodeling upon aging.

In addition to hypercholesterolemia induced by high-cholesterol feeding, other postnatal environmental risk factors are likely to promote more severe vascular disease. Therefore, exposure should be limited to maintain a relatively low disease risk throughout life. Awareness and development of risk reducing programs in adult life are also beneficial for a second reason; it reduces prenatal risk factors as well when applied to pregnant women and women in their reproductive period. General monitoring of for example cholesterol levels and

identification of markers of inflammation in relation to apoE polymorphisms may identify a high-risk population of pregnant women. To reduce the cardiovascular disease risk in progeny, lifestyle management programs and dietary intervention should be introduced for these women.

In apoE^{+/-} mice fetuses and neonates from both normocholesterolemic wild-type and hypercholesterolemic apoE^{-/-} mothers no hemodynamically induced atherosclerosis was detected throughout the vasculature. In contrast, in humans fatty streaks already develop in the fetal aorta.⁵ Development of these lesions is greatly enhanced in progeny from hypercholesterolemic mothers. In childhood, despite normal cholesterol levels, lesion progression is higher in these children.⁶ The apparent distinction in the rate of lesion development between mice and humans may be explained by hemodynamics. In mice, the average wall shear stress in the carotid artery is more than 6 times higher than in humans.¹⁷ Because high shear stress is athero-protective, mice may be more resistant to atherosclerosis than humans. We hypothesize that in human fetuses, the combined effects of epigenetic programming by maternal hypercholesterolemia, and the intrauterine environment almost directly initiate the process of atherosclerosis in the fetal vascular wall. Elucidation of the mechanisms of priming by maternal atherosclerotic risk factors would give more insight into the fetal tissues that are affected and may provide us with early indicators of atherosclerosis susceptibility (reviewed by DeRuiter and colleagues¹⁸).

In conclusion, intrauterine exposure to maternal apoE-deficiency, through epigenetic programming of the fetus, plays a key role in neointimal lesion initiation in adult offspring, even without high-cholesterol feeding. Hypercholesterolemia induced by high-cholesterol feeding promotes more severe vascular disease. Because there is increasing evidence that prenatal atherosclerotic risk factors are of importance for development of adult vascular disease, they should be taken into account in lifestyle management programs of pregnant women and mothers of the future.

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Chapter 4

The Effect of Maternal Hypercholesterolemia as a Consequence of LDL Receptor-Deficiency on Vascular Lesion Formation in the Offspring

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Abstract

In a recent study, we demonstrated that intrauterine exposure to maternal hypercholesterolemia, oxidative stress, and a pro-inflammatory immune status as a consequence of maternal apoE-deficiency damaged the fetal vasculature and accelerated neointima formation in adult offspring. To more specifically determine the role of maternal hypercholesterolemia in the process of intrauterine programming of adult cardiovascular disease, we aimed to address this issue in the low-density lipoprotein receptor-deficient ($Ldlr^{-/-}$) mouse model. Hypercholesterolemic $Ldlr^{-/-}$ mothers were crossbred with normocholesterolemic heterozygous $Ldlr$ -deficient fathers ($Ldlr^{+/-}$) and vice versa. In this way, we were able to study the effects of maternal hypercholesterolemia on both $Ldlr^{+/-}$ and the $Ldlr^{-/-}$ offspring. Analysis of the fetal vasculature and cholesterol levels of $Ldlr^{+/-}$ fetuses revealed no detrimental effects of maternal hypercholesterolemia. The long-term consequences of intrauterine exposure to maternal hypercholesterolemia were minimal as neointima formation, although a harsh Western-type diet was provided, could not be induced in $Ldlr^{+/-}$ offspring. Solely in spontaneous atherosclerosis in $Ldlr^{+/-}$ mice from $Ldlr^{-/-}$ mothers, a difference in aortic medial hypertrophy was detected in comparison to $Ldlr^{+/-}$ offspring from $Ldlr^{+/-}$ mothers. As expected, the $Ldlr^{-/-}$ offspring showed fetal intimal thickening and profound spontaneous atherosclerosis in the adult stage. We conclude that maternal hypercholesterolemia only minimally affects the heterozygous offspring. The increased susceptibility to develop atherosclerosis is not provoked in the $Ldlr$ -deficient mouse as compared to the apoE-deficient mouse.

Introduction

In a previous study, we have reported that intrauterine exposure to maternal apolipoprotein E-deficiency (apoE^{-/-}) induced a susceptibility for cardiovascular disease in heterozygous apolipoprotein E-deficient (apoE^{+/-}) offspring.¹ In apoE^{+/-} fetuses from apoE^{-/-} mothers, major endothelial damage was detected in the vasculature, whereas no abnormalities were found in genetically identical apoE^{+/-} fetuses from wild-type mothers. In adult apoE^{+/-} offspring from apoE^{-/-} mothers neointima formation was accelerated after challenging of the carotid arteries through induction of low shear stress levels by partial constriction of the vessel in combination with a high-cholesterol diet. Almost no lesions were found in offspring from wild-type mothers. Thus, our data demonstrated that maternal apoE-deficiency and/or environmental risk factors associated with apoE-deficiency prime genetically relatively insensitive apoE^{+/-} fetuses for accelerated cardiovascular disease in adult life.

ApoE plays an important role in lipoprotein metabolism by functioning as a ligand for receptor-mediated clearance of VLDL and chylomicron remnant lipoproteins.² ApoE-deficiency results in severe hypercholesterolemia,^{3,4} and is associated with increased oxidative stress,⁵ and a pro-inflammatory immune status.⁶ Furthermore, apoE is an important modulator of innate immune function.⁷ The apoE^{-/-} mouse model therefore presents a number of pro-atherogenic risk factors. We would like to further unravel which maternal atherogenic risk factor is responsible for the intrauterine programming, and the subsequent increase in susceptibility for atherosclerosis.

Hypercholesterolemia is considered to be one of the main risk factors for atherosclerosis in adult life. Hypercholesterolemia also occurs during the course of normal human pregnancy.^{8,9} The serum cholesterol levels of most women increase by approximately 25-50% throughout pregnancy. The lipid profile the women show in the third trimester would be considered highly atherogenic in a nonpregnant state. High circulating concentrations of cholesterol in the mother have been associated with a higher number and increased size of fatty streaks in the fetal aorta and augmented lesion progression in early childhood of the progeny.^{10,11} Studies in rabbits demonstrated similar outcomes.^{12,13} These data suggest that hypercholesterolemia can adversely influence cardiovascular development and promotes early atherogenesis in human and rabbit progeny. To further address the role of hypercholesterolemia in intrauterine programming of adult cardiovascular disease in a murine model, we used the low-density lipoprotein receptor deficient (Ldlr^{-/-}) mouse strain. Ldlr-deficiency results in profound elevation of circulating IDL

and LDL levels, total plasma cholesterol, and hyperresponsiveness to dietary cholesterol.^{14,15}

The present experiments were designed to assess the effects of maternal hypercholesterolemia per se on intrauterine programming of adult cardiovascular disease in mice offspring. We crossbred hypercholesterolemic *Ldlr*^{-/-} mothers with normocholesterolemic heterozygous *Ldlr*-deficient (*Ldlr*^{+/-}) fathers and vice versa. In this way, we were able to study the effect of maternal hypercholesterolemia on vascular development in both *Ldlr*^{+/-} and *Ldlr*^{-/-} fetuses and examine the long-term consequences on neointima formation in the carotid arteries, as well as on diet-induced spontaneous plaque development in the aortas of adult offspring. Unexpectedly, results indicated that profound maternal hypercholesterolemia has only modest effects on fetal vascular development and induced and spontaneous atherosclerosis in *Ldlr*^{+/-} offspring, in contrast to the expected abnormalities in the *Ldlr*^{-/-} offspring.

Methods

Mice

The $Ldlr^{-/-}$ mice were purchased from Jackson Laboratories (Wilmington, USA). The $Ldlr^{+/-}$ mothers were generated within our breeding facility by crossbreeding C57Bl/6J females (Charles River Laboratories, Maastricht, The Netherlands, import agency for Jackson Laboratories) with $Ldlr^{-/-}$ males. Both $Ldlr^{+/-}$ and $Ldlr^{-/-}$ mothers were fed a Western-type diet (0.25% cholesterol, Hope Farms, Woerden, The Netherlands) from 3 weeks before pregnancy onwards. The normocholesterolemic $Ldlr^{+/-}$ females were mated with $Ldlr^{-/-}$ males and the hypercholesterolemic $Ldlr^{-/-}$ females with $Ldlr^{+/-}$ males (Table 1). Through crossbreeding $Ldlr^{+/-}$ and $Ldlr^{-/-}$ fetuses, age embryonic day 17.5 (E17.5), were obtained from both $Ldlr^{+/-}$ and $Ldlr^{-/-}$ mothers (Table 1). Wild-type ($Ldlr^{+/+}$) fetuses from wild-type mothers were used as control. Each group consisted of 20 fetuses. Mouse genotypes were determined by PCR.

To induce postnatal hypercholesterolemia in female $Ldlr^{+/-}$ offspring from both $Ldlr^{+/-}$ and $Ldlr^{-/-}$ mothers (n=6 each group), a harsh Western-type diet was required (1% cholesterol and 0.5% cholate). Female $Ldlr^{-/-}$ mice from both groups (n=6 each group) were fed a Western-type diet with 0.25% cholesterol from weaning onwards. Diet and water were provided ad libitum. The Committee on Animal Welfare, Leiden University Medical Center, approved all of the animal experiments.

Plasma Measurements

Total plasma cholesterol and triglyceride levels were quantified by colorimetric assays (Roche, Almere, The Netherlands) in blood plasma of 4-hour fasted animals. In adult mice blood samples were obtained through tail bleeding while fetal blood samples were acquired by decapitation at E17.5. Maternal blood samples were acquired prior to dietary intervention, before pregnancy and at pregnancy day 17.5. In offspring plasma lipids were monitored at the postnatal ages of 4, 8 and 16 weeks.

Perivascular Collar Placement

Neointima formation was induced by perivascular collar placement around the left common carotid artery in $Ldlr^{+/-}$ offspring at the age of 16 weeks, as previously described.^{1,16} The extent of neointima formation was examined 4 weeks after the collar procedure.

Tissue Harvesting

Fetal thorax tissue

Fetal thorax tissue was obtained at E17.5. Fixation for 48 hours in 4% paraformaldehyde in 0.1 mol/L sodium phosphate buffer was followed by dehydration in series of ethanol and xylene. Subsequently, the specimens were embedded in paraffin. For cryosections, the thorax material was directly embedded in TissueTek (Bayer, Mijdrecht, The Netherlands) and stored at -20°C until use.

Adult carotid arteries and aorta

At the age of 20 weeks, *Ldlr*^{+/-} and *Ldlr*^{-/-} offspring were anesthetized. Pressure-perfusion (76 mm Hg) was performed through the cardiac left ventricle with sterile PBS for 5 minutes. The aorta and both common carotid arteries were excised and the collar around the left common carotid artery of *Ldlr*^{+/-} mice was removed. The carotid arteries and aortas were fixed in 4% paraformaldehyde in 0.1 mol/L sodium phosphate buffer for 6 and 48 hours, respectively, or directly embedded in TissueTek. Transverse 5-μm sections were cut and serially mounted.

Immunohistochemistry

Routine staining was performed with hematoxylin and eosin (HE), Resorcin-Fuchsin for detection of elastin, and Oil Red O on cryosections to detect lipid deposition. Immunohistochemistry was performed as described earlier^{1,17} with the primary antibodies rabbit polyclonal anti-von Willebrand factor (vWF, 1:2000, DAKO, Glostrup, Denmark), rat monoclonal anti-CD31 (1:50, PharMingen, Alphen, The Netherlands), and rat anti-ICAM-1 (1:250, GeneTex, Inc., San Antonio, USA) to examine endothelial cells. The CD31 signal was enhanced with a tyramide signal amplification system (DAKO). 3-3' Diaminobenzidine tetrahydrochloride was used as chromogen and brief counterstaining was performed with Mayer's hematoxylin.

Morphometric Analysis

In fetuses the volume of the aortic arch was estimated by using the Cavalieri principle,¹⁸ a point counting method using a grid. Ten equally spaced HE stained sections covering the complete arch were examined. In adult offspring total atherosclerosis, medial wall and lumen volume of the aortic arch were estimated in 20 equally spaced sections. Carotid artery medial volume was measured in 10 equally spaced sections covering a total length of 0.7 mm proximal to the collar.

Statistical Analysis

Data are represented as mean $\pm$ SEM. One-way ANOVA and the two-tailed Student's *t*-test were used to compare plasma cholesterol and triglycerides levels at several time points and between $Ldlr^{+/-}$ and $Ldlr^{-/-}$ mothers. The latter test was also used to evaluate differences between the two $Ldlr^{+/-}$ groups of fetuses and between both $Ldlr^{-/-}$ groups at E17.5 and analysis of the morphometric data. The differences were considered to be significant if $P < 0.05$.

Results

Maternal Lipid Levels

Western-type diet feeding of $Ldlr^{+/-}$ mothers slightly increased their plasma cholesterol levels, but hypercholesterolemia before pregnancy was only detected in $Ldlr^{-/-}$ mothers (Table 2). Significant differences were observed between total plasma cholesterol and triglycerides levels of $Ldlr^{+/-}$ and $Ldlr^{-/-}$ mothers at all experimental conditions: before dietary intervention, after addition of Western-type diet and at pregnancy day 17.5. Plasma cholesterol concentrations in $Ldlr^{+/-}$ mothers remained within normal range throughout pregnancy. As could be expected from the data on $apoE^{-/-}$ mice and other species such as rabbits,^{1,19} the total reduction in plasma cholesterol concentration from conception until pregnancy day 17.5 was striking in $Ldlr^{-/-}$ mothers, namely 62.1%. Nevertheless, hypercholesterolemia was still detected at pregnancy day 17.5 in these mothers.

Fetal Lipid Levels

To address the question whether profound maternal hypercholesterolemia influences fetal plasma cholesterol levels in late fetal development, we assessed the total plasma cholesterol and triglyceride levels in $Ldlr^{+/-}$ and $Ldlr^{-/-}$ fetuses at E17.5. Intrauterine exposure to maternal hypercholesterolemia did not add to the fetal cholesterol concentrations in $Ldlr^{+/-}$ fetuses. Comparable normocholesterolemic plasma cholesterol levels were observed between $Ldlr^{+/-}$ fetuses from $Ldlr^{+/-}$ mothers and those from $Ldlr^{-/-}$ mothers (Table 1).

In $Ldlr^{-/-}$ fetuses from $Ldlr^{+/-}$ mothers the cholesterol levels were similar to those observed in $Ldlr^{+/-}$ fetuses. The effects of maternal hypercholesterolemia on fetal lipid metabolism were only found in $Ldlr^{-/-}$ fetuses from $Ldlr^{-/-}$ mothers. Maternal hypercholesterolemia significantly enhanced plasma cholesterol concentrations in $Ldlr^{-/-}$ fetuses as compared to maternal normocholesterolemia (4.77 ± 1.14 versus 3.08 ± 0.26 mmol/L, $P < 0.000$). In a few cases, hypercholesterolemic concentrations were detected with a maximum of 10.6 mmol/L. Because of the large variation observed in fetal cholesterol concentrations in $Ldlr^{-/-}$ fetuses from $Ldlr^{-/-}$ mothers (2.7 mmol/L – 10.6 mmol/L), we examined the possible effect of the number of fetuses in one pregnancy and the positioning within the uterine horns. No correlation was found. A rise in fetal plasma cholesterol levels was accompanied by a small increase in triglyceride concentrations in $Ldlr^{-/-}$ fetuses from $Ldlr^{-/-}$ mothers, but the levels remained within range of the values detected in $Ldlr^{+/-}$ fetuses.

Table 1. Crossbreeding scheme and fetal lipid levels

ID	Maternal genotype	Paternal genotype	Maternal phenotype	Offspring	TC E17.5	TG E17.5
1	Ldlr ^{+/-}	Ldlr ^{-/-}	NC	Ldlr ^{+/-}	2.84 ± 0.14	0.49 ± 0.03
2	Ldlr ^{+/-}	Ldlr ^{-/-}	NC	Ldlr ^{-/-}	2.86 ± 0.16	0.50 ± 0.02
3	Ldlr ^{-/-}	Ldlr ^{+/-}	HC	Ldlr ^{+/-}	3.08 ± 0.26	0.50 ± 0.03
4	Ldlr ^{-/-}	Ldlr ^{+/-}	HC	Ldlr ^{-/-}	4.77 ± 1.14 [*]	0.66 ± 0.13

All data are shown as mean ± SEM. NC indicates normocholesterolemia; HC, hypercholesterolemia; TC, total plasma cholesterol (mmol/L); TG, total plasma triglycerides (mmol/L). ^{*}*P* < 0.000 versus Ldlr^{-/-} fetuses from Ldlr^{+/-} mothers.

Table 2. Maternal plasma cholesterol and triglyceride levels

	Ldlr ^{+/-}	Ldlr ^{-/-}
TC		
Before diet	2.37 ± 0.10	6.34 ± 0.21 [†]
Western-type diet	4.17 ± 0.14 [*]	35.73 ± 1.22 ^{*†}
Pregnancy day 17.5	2.67 ± 0.15 [*]	13.53 ± 1.35 ^{*†}
TG		
Before diet	0.49 ± 0.02	1.21 ± 0.06 [†]
Western-type diet	0.53 ± 0.02	3.13 ± 0.30 ^{*†}
Pregnancy day 17.5	0.54 ± 0.04	2.00 ± 0.14 ^{*†}

All data are shown as mean ± SEM. TC indicates total plasma cholesterol (mmol/L); TG, total plasma triglycerides (mmol/L). Significant differences (^{*}*P* = 0.000) were observed between TC levels before diet, after dietary intervention and at pregnancy day 17.5 in Ldlr^{+/-}, as well as in Ldlr^{-/-} mothers. In the latter group, TG levels also showed significant differences throughout the experiment. Comparison of TC and TG between Ldlr^{+/-} and Ldlr^{-/-} mothers revealed significant differences at all experimental conditions ([†]*P* < 0.000).

Fetal Vascular Morphology

Ldlr^{+/-} fetuses

We morphologically examined the fetal aortic arch and carotid arteries of Ldlr^{+/-} fetuses from Ldlr^{+/-} and Ldlr^{-/-} mothers to determine the effects of severe hypercholesterolemia on fetal vascular development. No damaging effect of intrauterine exposure to maternal hypercholesterolemia associated with Ldlr-deficiency could be detected in the endothelial cell layer in the carotid arteries and aortas of Ldlr^{+/-} fetuses. However, in comparison to Ldlr^{+/-} control fetuses, all Ldlr^{+/-} fetuses showed endothelial cell activation, characterized by reduced vWF as a result of exocytosis, and enhanced ICAM-1 staining (Figure 1A-B, D-E). Minimal traces of vWF were found in the subendothelial space. The endothelial cell lining and elastic laminae were intact in all arteries examined as shown by the CD31 and

Resorcin-Fuchsin staining (Figure 1G-H, J-K). No signs of vascular pathology were found in the aortas of $Ldlr^{+/+}$ and $Ldlr^{+/-}$ fetuses.

Ldlr^{-/-} fetuses

In $Ldlr^{-/-}$ fetuses from normocholesterolemic $Ldlr^{+/-}$ mothers, a phenotype similar to that seen in the $Ldlr^{+/-}$ fetuses (group 1 and 2) was observed. We expected that combined genetic susceptibility to cardiovascular disease and maternal hypercholesterolemia would cause clear damage in the vasculature of $Ldlr^{-/-}$ fetuses from $Ldlr^{-/-}$ mothers. Besides a few small areas of intimal thickening, consisting of several cells and an additional elastic lamina, no abnormalities were detected in the intima of the carotid arteries in all mice (Figure 1C,F,I,L). In the vascular media, a more spacious distribution of the elastic lamina and medial presence of vWF was shown (Figure 1F,L).

In the ascending aorta of all $Ldlr^{-/-}$ fetuses from hypercholesterolemic $Ldlr^{-/-}$ mothers, however, endothelial detachment and intimal thickening were observed relative to normal morphology in $Ldlr^{+/-}$ and $Ldlr^{+/+}$ fetuses (Figure 2A-C). In the aortic arch fewer abnormalities were observed than in the ascending aorta and in the descending aorta normal morphology was shown. Irregular distribution of the elastic lamina in the media was found (Figure 2D-F). In addition, the orientation of the spindle-shaped smooth muscle cells in the media as present in normal aortas was disturbed in aortas of intrauterine exposed $Ldlr^{-/-}$ fetuses (Figure 2G-I). VWF was present in endothelial cells, as well as in the subendothelial intimal and medial layers. No free lipid deposition and fatty streaks were observed. No clear link could be established between the plasma cholesterol levels and the severity of intimal thickening in the ascending aortas of the $Ldlr^{-/-}$ fetuses.

As there seemed to be a more spacious distribution of elastic lamina and intimal thickening in the carotid arteries and ascending aorta of $Ldlr^{-/-}$ fetuses from $Ldlr^{-/-}$ mothers, we estimated the medial wall volume of the ascending aorta and aortic arch by morphometric analysis. The aortic medial wall volumes of intrauterine exposed $Ldlr^{-/-}$ fetuses were not enhanced compared with $Ldlr^{-/-}$ fetuses from $Ldlr^{+/-}$ mothers (0.053 ± 0.003 versus 0.053 ± 0.010 mm³) thereby excluding fetal vascular hypertrophy. Furthermore, no differences were observed between the medial volumes of $Ldlr^{-/-}$ and $Ldlr^{+/-}$ fetuses. (Group 1 0.054 ± 0.008 versus group 2 0.071 ± 0.002 mm³). All estimated volumes were comparable to $Ldlr^{+/+}$ controls (0.059 ± 0.016 mm³).

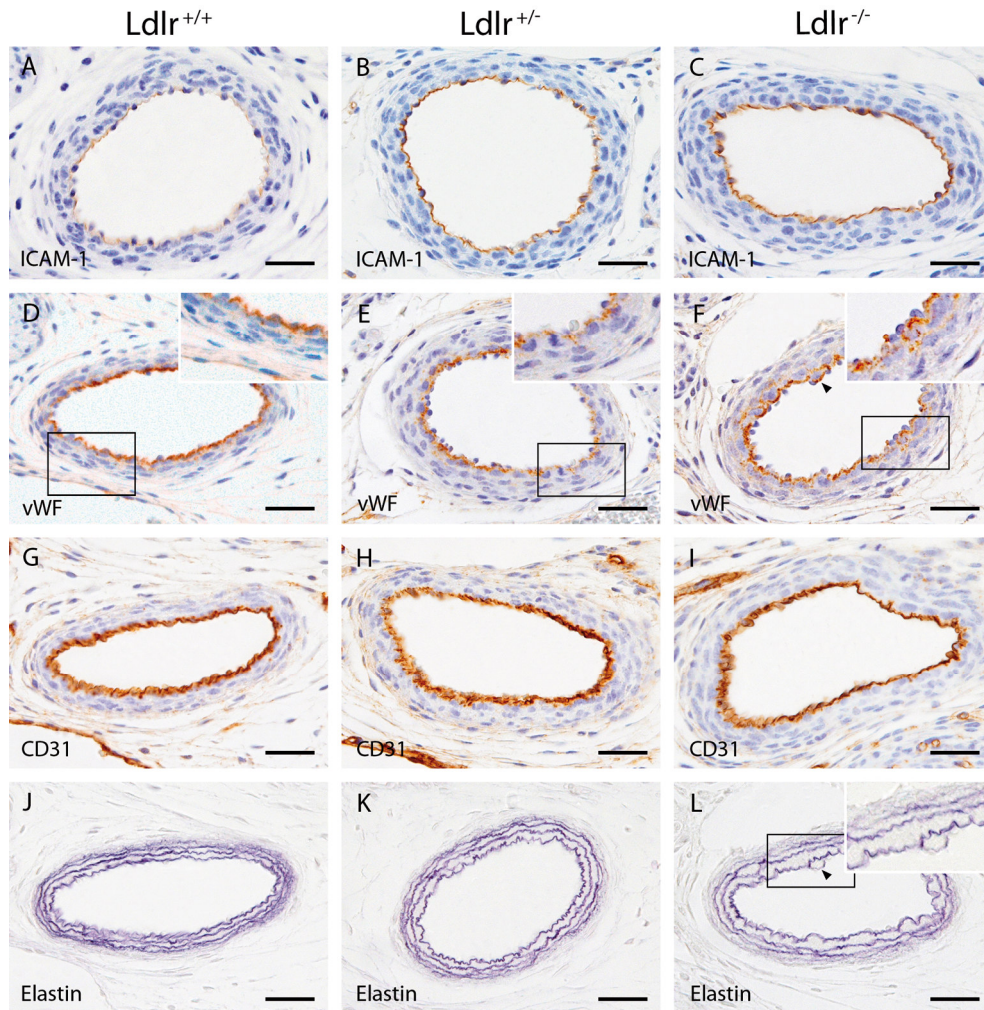


Figure 1. Morphology of fetal carotid arteries. Cross-sections from (A,D,G,J) $Ldlr^{+/+}$, (B,E,H,K) $Ldlr^{+/-}$ from $Ldlr^{+/-}$ mothers and (C,F,I,L) $Ldlr^{-/-}$ fetuses from $Ldlr^{-/-}$ mothers at E17.5. Because identical staining patterns were found in $Ldlr^{+/-}$ from $Ldlr^{-/-}$ mothers and $Ldlr^{-/-}$ from $Ldlr^{+/-}$ mothers, sections B,E,H,K also represent these two groups. Immunostaining for (A-C) ICAM-1, (D-F) vWF, (G-I) CD31 and (J-L) elastin. Picture 1D,E,F,L show enlargement of box in right upper corner. Arrows indicate intimal thickening, which was observed in the carotid arteries of all $Ldlr^{-/-}$ fetuses from $Ldlr^{-/-}$ mothers. Scale bars: 30 μ m.

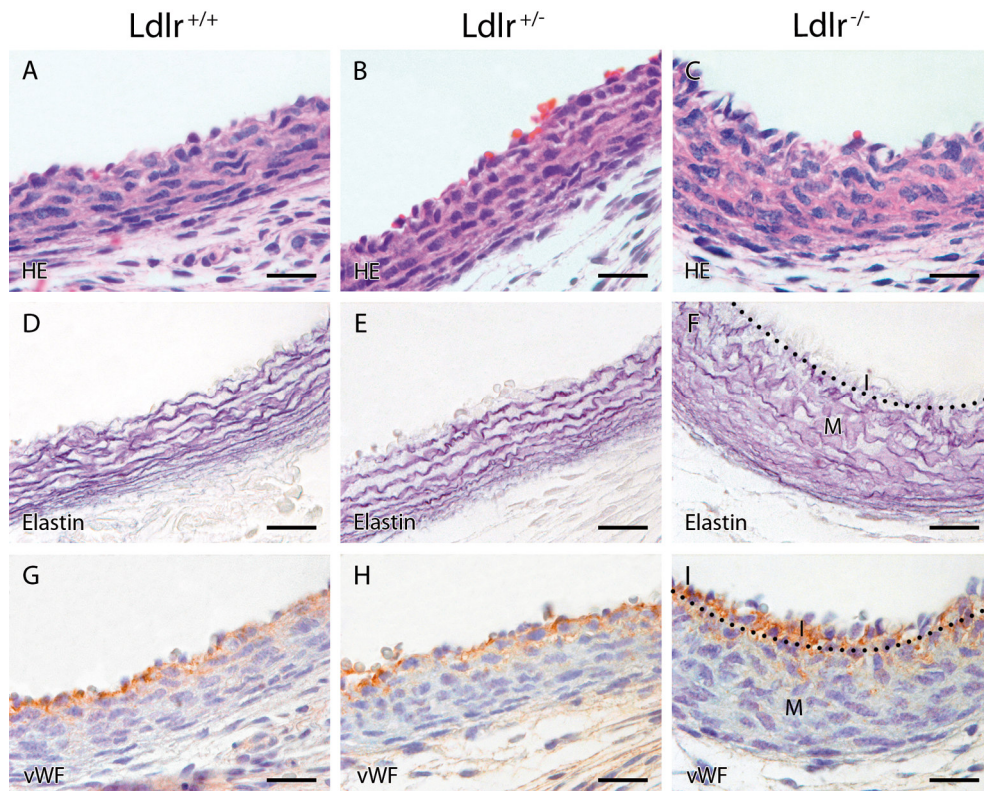


Figure 2. Morphology of fetal ascending aorta. Cross-sections from (A,D,G) $Ldlr^{+/+}$, (B,E,H) $Ldlr^{+/-}$ from $Ldlr^{-/-}$ mothers and (C,F,I) $Ldlr^{-/-}$ fetuses from $Ldlr^{-/-}$ mothers with (A-C) general HE staining, (D-F) elastin and (G-I) anti-vWF. Section B,E,H also represent $Ldlr^{+/-}$ from $Ldlr^{+/-}$ and $Ldlr^{-/-}$ from $Ldlr^{+/-}$ mothers. Note the endothelial detachment, intimal thickening and medial disorganization as observed in all the $Ldlr^{-/-}$ fetuses from $Ldlr^{-/-}$ mothers. I indicates intima; M, media and dotted line represents border between intima and media. Scale bars: 20 μ m.

Induction of Neointima Formation in $Ldlr^{+/-}$ Offspring

To assess the effect of in utero exposure to $Ldlr$ -deficiency on lesion initiation in adult life, a perivascular constrictive collar was placed around the carotid artery of $Ldlr^{+/-}$ offspring from $Ldlr^{+/-}$ and $Ldlr^{-/-}$ mothers. From weaning onwards all $Ldlr^{+/-}$ animals received a Western-type diet containing 1% cholesterol and 0.5% cholate. Such a harsh diet was necessary to induce postnatal hypercholesterolemia. No differences were observed between the total plasma cholesterol levels of the two groups throughout the experiment. Prior to collar placement, plasma cholesterol levels were 19.00 ± 1.54 and 19.58 ± 1.94 mmol/l, respectively. In spite of the high circulating cholesterol concentrations, 4 weeks after collar placement no neointimal

lesions were found. Moreover, no differences were found in the carotid medial volume of both groups of $Ldlr^{+/-}$ mice (0.019 ± 0.001 versus 0.016 ± 0.001 mm³).

Spontaneous Atherosclerosis Development in $Ldlr^{+/-}$ and $Ldlr^{-/-}$ Offspring

The two types of Western-type diet initiated spontaneous atherosclerosis development in the aortic arch of all animals. Lesions were detected in the aortic root, the inner curvature of the aortic arch and in the areas upstream of the branch points (Figure 3A). Intrauterine exposure of $Ldlr^{+/-}$ and $Ldlr^{-/-}$ offspring to maternal hypercholesterolemia did not affect the degree of advanced atherosclerosis in adult life as no differences were found in the total volumes of spontaneous atherosclerosis in the aortic arch between both groups of $Ldlr^{+/-}$ mice and between the two $Ldlr^{-/-}$ groups (Figure 3B). The only observed difference was found in the total volume of the vascular media of the aortic arch of $Ldlr^{+/-}$ mice from $Ldlr^{+/-}$ mothers. The volume was significantly increased compared with $Ldlr^{+/-}$ progeny from $Ldlr^{-/-}$ mothers (1.00 ± 0.05 versus 0.71 ± 0.07 mm³, $P = 0.016$, Figure 3C). Vascular lumen volume was maintained (1.66 ± 0.14 versus 1.69 ± 0.08 mm³).

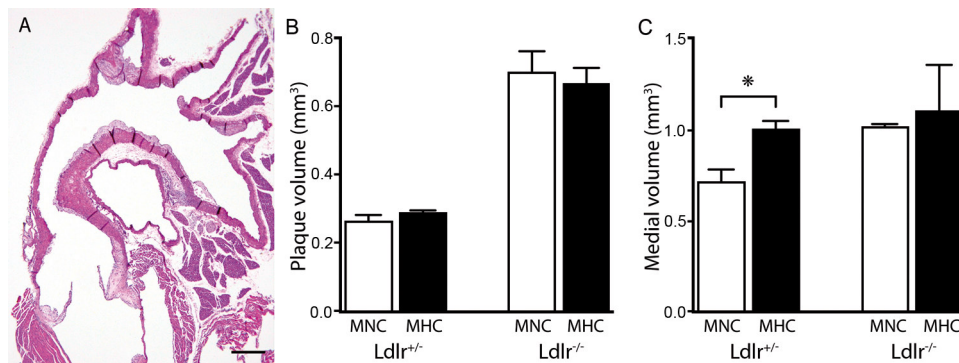


Figure 3. Effects of in utero exposure of $Ldlr^{+/-}$ and $Ldlr^{-/-}$ mice on spontaneous atherosclerosis in adult life. (A) Representative cross-section of the adult aorta showing the sites of atherogenesis; the aortic root, the inner curvature of the aortic arch and the areas upstream of the branch points. Scale bar: 300 μ m. Morphometric analysis and estimation of the aortic (B) plaque volume and (C) medial volume of $Ldlr^{+/-}$ and $Ldlr^{-/-}$ mice. MNC indicates maternal normocholesterolemia; MHC, maternal hypercholesterolemia. Data are mean $\pm$ SEM (n=6 each group). * $P < 0.05$.

Discussion

Studies in humans and rabbits have demonstrated that intrauterine exposure to maternal hypercholesterolemia enhances atherosclerotic lesion formation in the offspring prenatally and postnatally.¹⁰⁻¹³ In the present study, we investigated the role of maternal hypercholesterolemia associated with Ldlr-deficiency in the process of intrauterine programming of cardiovascular disease in mice. Whereas we expected that severe maternal hypercholesterolemia would affect vascular development in Ldlr^{+/-} fetuses in a similar way as observed in apoE^{+/-} mice,¹ the opposite was true. No harmful effects of maternal hypercholesterolemia were found on plasma cholesterol levels and the vasculature in Ldlr^{+/-} fetuses. In adult life, profound hypercholesterolemia in combination with the application of athero-prone low shear stress in the carotid arteries did not reveal any effect of intrauterine exposure on neointima formation. Solely in the process of spontaneous atherosclerosis, a significant increase in aortic medial hypertrophy was detected compared with Ldlr^{+/-} offspring from Ldlr^{+/-} mothers. The data indicate that the sum of pathogenic factors in Ldlr^{+/-} offspring is not sufficient, at least in this mouse strain, to cause atherosclerosis. The most apparent effects of maternal hypercholesterolemia were observed in Ldlr^{-/-} fetuses that are genetically susceptible to atherosclerosis. In these fetuses plasma cholesterol levels were significantly increased and already at this early point in life intimal thickening was detected in the aorta. However, the long-term consequences of prenatal exposure to hypercholesterolemia on the development of atherosclerosis were minimal in Ldlr^{-/-} offspring. Overall, the results suggest that the effect of severe maternal hypercholesterolemia on intrauterine programming of adult cardiovascular disease in mice is limited.

Despite much lower cholesterol levels in apoE^{-/-} mothers, major endothelial damage was detected in the vasculature of apoE^{+/-} fetuses in our previous study.¹ In moderately hypercholesterolemic adult apoE^{+/-} offspring from apoE^{-/-} mothers, severe neointima formation was detected after induction with a perivascular collar. Our data show that the atherosclerotic risk factors associated with maternal Ldlr-deficiency were insufficient to directly damage the vessels in Ldlr^{+/-} fetuses. This may have caused the lack of neointimal lesions in hypercholesterolemic adult Ldlr^{+/-} offspring from Ldlr^{-/-} mothers. The dual deficiency of both maternal and fetal Ldlr was necessary to observe a detrimental effect on the fetal vascular wall. This suggests that the combination of risk factors is crucial in determining whether or not the fetal vasculature is primed for accelerated atherosclerosis in adult life.

Besides the intrauterine environment, postnatal environmental triggers, as well as the chosen end points have to be evaluated. In spite of harsh Western-type diets inducing severe postnatal hypercholesterolemia, no effects of maternal

hypercholesterolemia were found on spontaneous atherosclerosis in $Ldlr^{+/-}$ and $Ldlr^{-/-}$ offspring. These data are partially in line with a study of Madsen and colleagues²⁰ who demonstrated that maternal apoE-deficiency during pregnancy did not aggravate the development of diet-induced advanced atherosclerosis in offspring aged 24 weeks compared with nonexposed counterparts. Napoli and colleagues reported that in chow fed $Ldlr^{-/-}$ offspring from hypercholesterolemic mothers postnatal atherogenesis was enhanced at the age of 12 weeks relative to progeny of normocholesterolemic mothers.²¹ We propose that the postnatal use of harsh Western-type diets instead of chow concealed the effects of in utero priming on spontaneous atherosclerosis development in the $Ldlr^{+/-}$ and $Ldlr^{-/-}$ offspring. Moreover, lesion examination during the initial phases of atherogenesis may potentially reveal more differences in lesion acceleration between the groups than in the stage of advanced lesion formation as the latter can be considered a feature of the plateau phase of the process.

Recent studies demonstrated that besides hypercholesterolemia, oxidative stress and inflammatory pathways play an important role in intrauterine programming of adult disease. Maternal immunization of athero-prone New Zealand White rabbits and $Ldlr^{-/-}$ mice with oxidized LDL reduced development of atherosclerosis in offspring independent of maternal cholesterol status during pregnancy.²² In addition, maternal treatment with vitamin E, an antioxidant, or cholestyramine, a lipid-lowering drug, significantly diminished atherosclerosis in the rabbit offspring suggesting a role for oxidative stress and inflammatory pathways.^{12,13} A striking observation in our study was the lack of induction of neointima in the presence of high circulating cholesterol concentrations. Obviously, intrauterine exposure, postnatal hypercholesterolemia and athero-prone low shear stress together are not sufficient to trigger neointima formation in the carotid arteries of $Ldlr^{+/-}$ mice. Because in both the apoE^{+/+} and $Ldlr^{+/-}$ mouse models hypercholesterolemia appears not to be the most important trigger for prenatal programming, we hypothesize that other risk factors associated with apoE-deficiency play an important role in the process of programming of adult atherosclerosis. These could be murine apoE itself, maternal inflammation, and/or adaptive immunity.

Intrauterine exposed $Ldlr^{+/-}$ mice showed a more proliferative response of smooth muscle cells in the development of spontaneous atherosclerosis compared with $Ldlr^{+/-}$ mice from $Ldlr^{+/-}$ mothers. These data suggest that maternal hypercholesterolemia influences adult susceptibility to atherosclerosis of $Ldlr^{+/-}$ offspring by affecting smooth muscle cells. Increasing evidence from studies using animal models indicates that permanent epigenetic changes, through e.g. DNA methylation or chromatin modifications, could be responsible for the in utero

programming of increased atherosclerosis risk in adult life (reviewed by DeRuiter and colleagues²³). Further studies are necessary to elucidate the exact signals and mechanisms through which hypercholesterolemia influences the endothelial cells and smooth muscle cells in the process of priming.

In conclusion, the present study demonstrates that severe maternal hypercholesterolemia caused by *Ldlr*-deficiency has only modest effects on fetal vascular development and atherosclerosis in *Ldlr*^{+/-} and *Ldlr*^{-/-} offspring. The data indicate that maternal hypercholesterolemia is sufficient for intrauterine programming of adult cardiovascular disease in mouse models. However, this does not preclude that hypercholesterolemia has a modulating role in this process. Whether maternal apoE status, inflammation and/or innate immunity play a dominant role in prenatal programming of atherosclerosis remains to be investigated.

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Chapter 5

Endothelial-to-Mesenchymal-Transformation during the Initial Stages of Neointima Formation

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Endothelial-to-Mesenchymal-Transformation during the Initial Stages of Neointima Formation

Abstract

In a previous study we demonstrated that embryonic endothelial cells in vivo have the capacity to undergo endothelial-to-mesenchymal-transformation (EMT). We hypothesized that adult endothelial cells also have that capacity in vivo during the initial phase of neointimal lesion development. To investigate this, we induced neointimal lesions, a reflection of cardiovascular disease, in Tie2LacZ mice by placement of a nonconstrictive collar around the femoral artery. Three days after collar placement we detected endothelial detachment and proliferation. In addition, a decrease in phosphoSmad2 staining, a Tgf- β -related protein associated with differentiation and quiescence, was observed. From 7 days onwards β -galactosidase positive mesenchymal cells were found in the developing neointimal lesions. A number of these β -galactosidase-positive mesenchymal cells also showed positivity for α -smooth muscle actin. Since Tie2 is also expressed in part of the bone marrow cells, we assessed their contribution to neointimal lesion formation. Beta-galactosidase-positive bone marrow-derived cells were recruited to the adventitia, but could not be detected in the neointima and media. We conclude that adult vascular endothelial cells in vivo have the capacity to undergo EMT. Furthermore, through EMT, endothelial cells contributed to the total pool of mesenchymal cells that compose the neointima. These data indicate that EMT could be important in development and progression of cardiovascular disease.

Introduction

In a previous study using a quail model, we demonstrated that embryonic endothelial cells *in vivo* have the capacity to undergo endothelial-to-mesenchymal-transformation (EMT).¹ During the initial phases of arterial differentiation, embryonic endothelial cells of the descending aorta detached from the epithelial layer and differentiated into subendothelial mesenchymal cells. The transformation from endothelial to mesenchymal phenotype was accompanied by a reduction in the expression of the endothelial marker QH1 and gain of α -smooth muscle actin positivity. A number of reports substantiated our data and appointed a role for Tgf- β signaling in this process.²⁻⁴ However, it remained unclear whether also adult endothelial cells have the potential to transdifferentiate into mesenchymal cells *in vivo*.

Therefore, in this study we investigated the capacity of adult vascular endothelial cells to undergo EMT *in vivo*. For this purpose we used Tie2LacZ reporter mice.⁵ The Tie2 gene encodes a vascular endothelial-specific receptor.^{6,7} Subsequently, in Tie2LacZ reporter mice positivity for β -galactosidase in the normal vascular wall is exclusively found in the endothelial cell population. Adult vascular endothelial cells are fully differentiated and are in a state of quiescence. To study the capacity of these cells to undergo EMT, we challenged the mouse vascular system by placement of a nonconstrictive collar around the femoral artery. This technique is frequently used to induce neointimal lesions, a reflection of cardiovascular disease.⁸⁻¹² Besides determination of the EMT capacity of adult endothelial cells *in vivo*, this experimental set-up enabled us to assess whether endothelial cells are active contributors to the mesenchymal cell population in neointimal lesions.

To investigate the role of Tgf- β signaling in our model, the expression patterns of several proteins of the Tgf- β /Smad signal transduction pathway¹³ were studied. Tgf- β can signal via two different pathways on endothelial cells. Tgf- β signaling through the type I receptor Alk5 and Smad2/3 has been reported to maintain a quiescent differentiated phenotype of the endothelium.¹⁴ Signaling through the Tgf- β type I receptor Alk1 and Endoglin via Smad1/5/8 on the other hand, stimulates proliferation and migration of endothelial cells.¹⁵

Our results demonstrate that adult endothelial cells have the capacity to undergo EMT *in vivo*. Furthermore, we show that a first population of mesenchymal cells in neointimal lesions is of endothelial cell origin.

Methods

Mice

For these experiments we used Tie2LacZ, Rosa26LacZ and wild-type FVB mice. The Tie2LacZ reporter line (a generous gift of Dr. U. Deutsch) was derived from founder 182.30 as described.⁵ The construct contained the 2.1 kb *HindIII* Tie2 promoter fragment followed by the LacZ reporter gene and simian virus 40 poly(A) signal sequence. In addition, a mouse Tie2 genomic fragment extended from an NgoMI site at the 3' end of exon 1 approximately 10 kb into the first intron downstream of the LacZ cassette (HHNS construct). The founders were backcrossed to FVB for more than 10 generations. The Rosa26LacZ reporter mouse, a knock-in mouse expressing the LacZ gene in essentially all tissues, was kindly provided by Dr. P. Soriano.¹⁶ Wild-type FVB mice were generated by our internal breeding facility. Diet and water were provided ad libitum. The Committees on Animal Welfare, Leiden University Medical Center and TNO Quality of Life, approved all animal experiments.

Femoral Artery Collar Placement

In Tie2LacZ mice average age 6 months, a nonconstrictive collar was placed around the femoral artery to induce neointima formation.^{8,9} With this technique neointimal lesions induced within the area of the collar. The mice were anesthetized by an intraperitoneal injection of 2.5 µl/g Hypnorm® (containing fentanyl citrate 0.315 mg/mL and fluanisone 10 mg/mL, Bayer, Mijdrecht, The Netherlands) and 2.5 µl/g Dormicum® (midazolam 5 mg/mL, Roche, Woerden, The Netherlands). The nonconstrictive polyethylene collar (0.40 mm inner diameter, 0.80 mm outer diameter, 1.5 mm length, Portex, Hythe, UK) was placed around one of the femoral arteries avoiding damage to the vascular wall. The other hind leg was sham-operated.

Beta-Galactosidase Staining

Tie2LacZ mice were sacrificed at 3, 7, 14 and 21 days (n=7, 16, 27 and 4) and the bone marrow recipients 14 days after collar placement, respectively. After cervical dislocation both collared and sham-operated femoral arteries were harvested. For detection of the β -galactosidase by light microscopy, tissues were first fixed in 2% paraformaldehyde (PFA) in phosphate buffered saline (PBS) for 2 hours at 4°C followed by incubation for 2 hours at 37 °C in a solution containing 10 mM potassium ferricyanide, 10 mM potassium ferrocyanide, 2 mM MgCl₂ and 0.1%

X-gal (5-bromo-4-chloro-3-indolyl-beta-D-galactopyranoside, Roche). Postfixation was performed overnight in 4% PFA after which the tissue was dehydrated in series of ethanol and xylene, and paraffin embedded.

For visualization of the β -galactosidase staining reaction by transmission electron microscopy the arteries were fixed in 2% PFA/0.1% glutaraldehyde in 0.1M cacodylate buffer (pH 7.4) for 1 hour at 4 °C followed by incubation in a solution containing 10 mM potassium ferricyanide, 10 mM potassium ferrocyanide, 2 mM $MgCl_2$ and 0.1% Bluo-gal (5-bromo-3-indolyl-beta-D-galactosidase, Invitrogen, Breda, The Netherlands) for 2 hours at 37 °C. Postfixation was carried out for 12 hours in 2% PFA/2% glutaraldehyde in 0.1M cacodylate buffer (pH 7.4) and in 1% OsO_4 /PBS for 1 hour. The tissue was embedded in epoxy resin mixture and sections of 1 μ m were cut. Sections of interest were re-embedded, ultra thin-sectioned and subsequently stained with lead citrate and uranyl acetate. The EM410 transmission electron microscope (Philips, Eindhoven, The Netherlands) was used for assessment of the β -galactosidase staining.

Immunohistochemistry

Overnight fixation of the tissues in 2% acetic acid in 98% ethanol or in 3% PFA in 66% ethanol was followed by dehydration in series of ethanol and xylene and the tissues were paraffin embedded. Transverse 5- μ m sections were cut and serially mounted. Unless indicated otherwise the immunohistochemistry was performed as previously described.¹⁷ Sections were incubated overnight at room temperature with rat monoclonal antibodies against ICAM-1 (1:100, R&D systems, Abingdon, UK), CD31 (1:500, Serotec, Düsseldorf, Germany), CD34 (1:50, PharMingen, Breda, The Netherlands), and Mac-3 (1:400, PharMingen). The rabbit polyclonal antibodies against phosphoSmad1/5/8 (PSmad1/5/8; 1:50) and phosphoSmad2 (PSmad2; 1:1500) were generated as previously described.¹⁸ Furthermore, rabbit polyclonal antibody against Von Willebrand factor (vWF; 1:500, DAKO, Glostrup, Denmark) and goat polyclonal antibodies against VCAM-1 (1:200, Santa Cruz, Heerhugowaard, The Netherlands) and Ki67 (1:1000, Sanver Tech, Heerhugowaard, The Netherlands) were used. Rabbit anti-rat biotin conjugate (1:200, Vector, Amsterdam, The Netherlands), goat anti-rabbit biotin conjugate (1:200, Vector) or rabbit anti-goat biotin conjugate (1:200, Vector) with normal rabbit and goat serum diluted in PBS were used as secondary antibodies and incubated for 1 hour at room temperature. Biotin labeling was followed by incubation with Vectastain ABC (Vector) for 1 hour. 3-3' diaminobenzidine tetrahydrochloride was used for visualization and counterstaining was performed with Mayer's hematoxylin.

For staining of the sections with mouse monoclonal anti- α smooth muscle actin (1:2000, Sigma-Aldrich), the primary antibody was coupled overnight to polyclonal rabbit anti-mouse peroxidase conjugate (1:200, DAKO) secondary antibody.¹⁹ Sections were incubated for 2 hours at room temperature. Several X-gal sections were stained with nuclear red using the standard protocol.

Bone marrow transplantations

To demonstrate that the neointimal cells expressing X-gal, Bluo-gal and α -smooth muscle actin were not derived from circulating cells expressing Tie2, the bone marrow of wild-type mice was replaced by bone marrow of Tie2LacZ and Rosa26LacZ mice and followed by collar placement 4 weeks after transplantation.

The drinking water of wild-type bone marrow recipients (n=8) was supplemented with the antibiotics polymyxin B (Bupha, Uitgeest, The Netherlands), ciprofloxacin (Bayer), amfotericine B (Bristol-Myers Squibb, Woerden, The Netherlands) and saccharose from week -1 until +4 after bone marrow transplantation. Donor bone marrow was harvested from femurs and tibias of Tie2LacZ (n=4) and Rosa26LacZ (n=4) mice. Bone marrow recipients were irradiated with a single dose of 13 Gy X-ray total body irradiation as previously described.²⁰ One day after irradiation approximately 1×10^7 donor cells were injected via caudal vein injection.

Statistical analysis

The endothelial cell proliferation response was studied in femoral arteries 3 days after collar placement or sham-operation (n=6 each group). The total number of nuclei and the total number of Ki67-positive nuclei were determined by counting the nuclei in each fifth section of 5 μ m over a length of 0.7 mm. The lumen volume was estimated by applying Cavalieri's principle²¹ and used to calculate and correct for endothelial surface area. Data are represented as mean $\pm$ SEM. Statistical significance of the differences between the groups were determined using the Student's *t*-test and considered significant if $P < 0.05$.

Results

Time Course of Neointima Formation

We studied the neointimal response at 3, 7, 14 and 21 days after collar placement and sham-operation. Normal vascular morphology was observed in the femoral arteries of sham-operated mice at all time points after intervention (Figure 1A). Similar morphology was detected in areas outside the region of the collar. Within the collar region on the other hand, 3 days after collar placement an endothelial cell response was observed characterized by detachment and proliferation (Figure 1B). Morphometric analysis based on the Ki67 staining revealed a significantly increased proliferation rate of the endothelial cells in collared femoral arteries compared with sham-operated femoral arteries (35.05 ± 6.09 versus $1.44 \pm 0.61\%$ Ki67-positive cells, $P < 0.001$, Figure 1C).

One layer of subendothelial cells was present after 7 days and 2 to 3 layers could be detected 14 days after collar placement. Endothelial cells of both collared and sham-operated femoral arteries showed positivity for vWF (Figure 2A-B). In addition to positivity in the endothelial cell lining, vWF deposition was found in the neointima of collared femoral arteries (Figure 2B). ICAM-1 expression in endothelial cells of collared femoral arteries was decreased compared with sham-operated arteries (Figure 2C-D). The CD31 staining remained unaffected and was restricted to the endothelial cells throughout the process of neointima development (Figure 2E-F). From 14 days onwards the first signs of fragmentation of the internal elastic lamina were observed and medial smooth muscle cells started to migrate into the neointima. After 21 days large parts of the internal elastic lamina had disappeared and many smooth muscle cells were present in the neointima (data not shown).

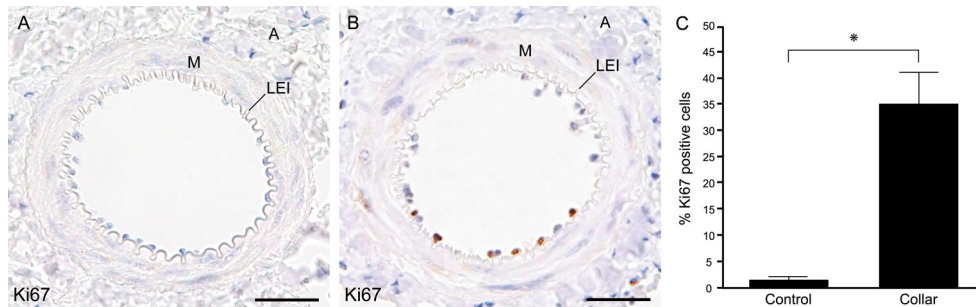


Figure 1. Endothelial cell proliferation 3 days after collar placement. Representative immunostaining for Ki67 in (A) sham-operated and (B) collared femoral arteries. LEI indicates lamina elastica interna; M, media; A, adventitia. Scale bar: 30 μ m. (C) Morphometric analysis of endothelial cell proliferation in the region of the collar compared with sham-operated femoral arteries. Data are means $\pm$ SEM, * $P < 0.001$.

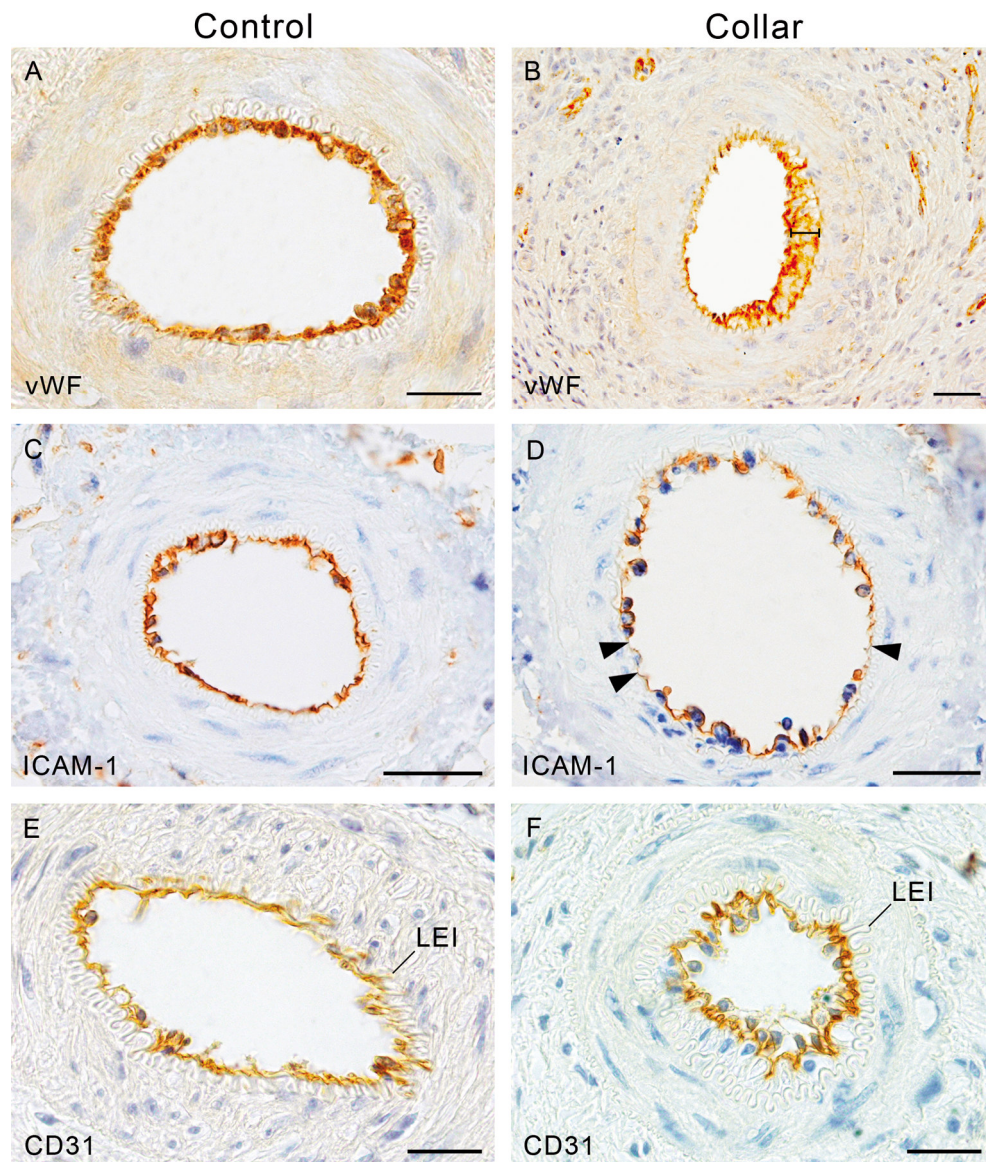


Figure 2. Endothelial cell markers in representative cross-sections from (A,C,E) sham-operated and (B,D,F) femoral arteries 7 days after collar placement that were stained with antibodies against (A-B) vWF, (C-D) ICAM-1 and (E-F) CD31. LEI indicates lamina elastica interna. Arrowheads show regions of endothelial cell detachment. Scale bars: A,E,F 20 µm, B,C,D 30 µm.

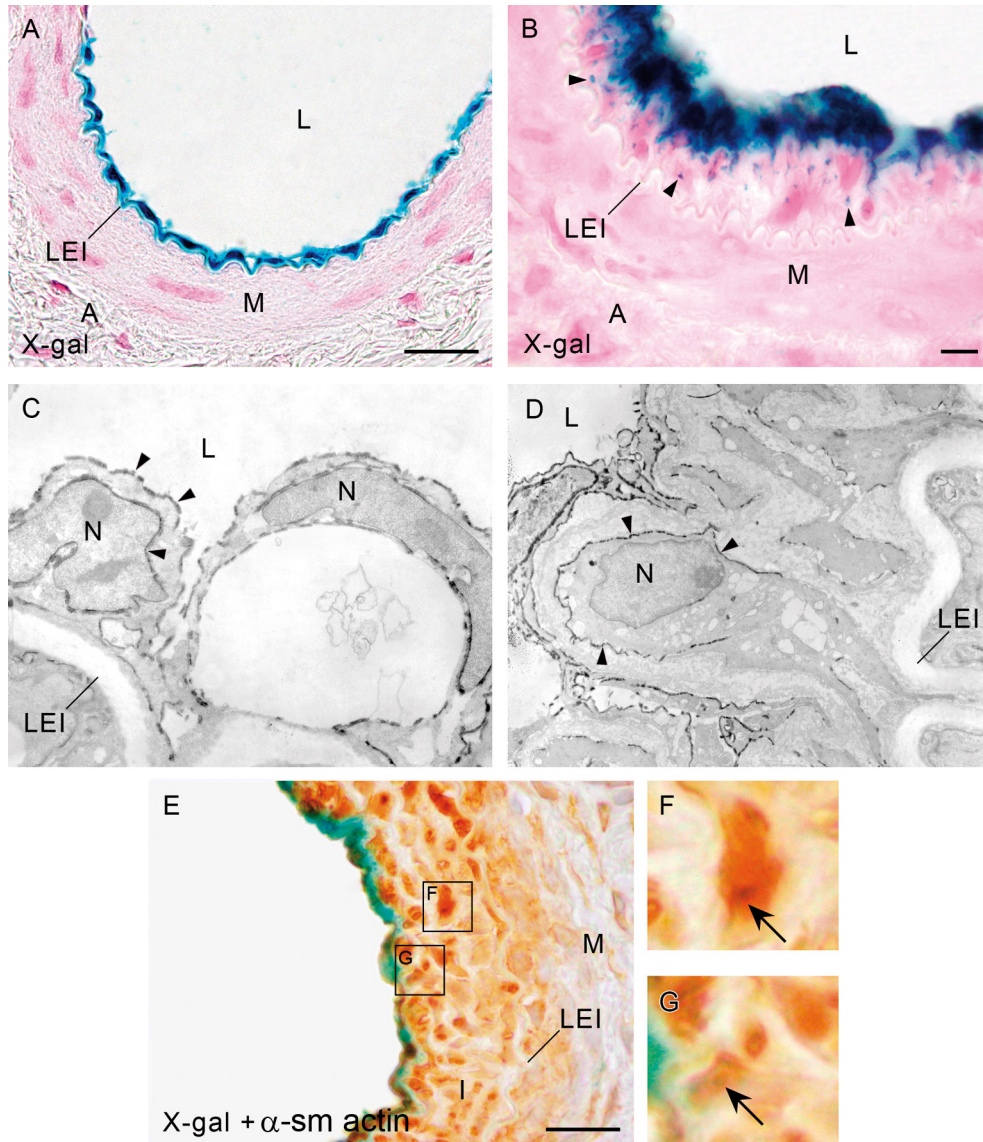


Figure 3. Beta-galactosidase expression in femoral arteries. Representative cross-sections from (A,C) sham-operated and (B,D,E) collared femoral arteries 2 weeks after collar placement. Beta-galactosidase expression in (A) endothelial cells and (B, small speckles, arrowheads) β -galactosidase expression in endothelial cells and mesenchymal cells. Transmission electron microscopic pictures of β -galactosidase in nuclear and cellular membranes of (C) endothelial cells and (D) cellular membranes of mesenchymal cells, magnification 5900x and 4400x respectively. (E) Beta-galactosidase and α -smooth muscle actin-positivity in mesenchymal cells. (F,G, α -sm actin) Detail of mesenchymal cells with α -sm actin and β -galactosidase within the same cell (arrow). L indicates lumen; LEI, lamina elastica interna; M, media; A, adventitia; N, nucleus. Scale bars: 20 μ m.

Endothelial-to-Mesenchymal-Transformation

In Tie2LacZ reporter mice, β -galactosidase can be detected in tissues expressing Tie2, such as vascular endothelial cells (Figure 3A). Medial smooth muscle cells and adventitial fibroblasts of the vascular wall on the other hand, were completely devoid of staining. To precisely determine the localization of X-gal precipitate in the endothelial cells, expression was studied with transmission electron microscopy. In endothelial cells, the X-gal precipitate was found in the nuclear and cellular membranes (Figure 3C). Furthermore, staining was observed in the Golgi of these cells. There was no indication of phagocytosis as neither vacuoles nor α -specific depositions of β -galactosidase were found.

From 7 days after collar placement onwards, β -galactosidase was not only detected in the endothelial cells, but also in the neointima (Figure 3B). In these mesenchymal cells β -galactosidase expression was solely observed in the cellular membrane (Figure 3D). Staining of X-gal in the mesenchymal cells gradually diminished but could still be detected at 14 days after collar placement. A number of β -galactosidase-positive mesenchymal cells showed positivity for α -smooth muscle actin from 7 days onwards (Figure 3E-G).

The results indicate that endothelial cells have the capacity to undergo EMT. However, Tie2 is not exclusively expressed in vascular endothelial cells, but can also be detected in part of the bone marrow-derived cells. To exclude the contribution of bone marrow-derived cells to the neointimal lesions, we performed bone marrow transplantations from Tie2LacZ and ROSA26LacZ mice to wild-type recipients prior to collar placement. No β -galactosidase-positive cells were detected in the endothelial cell lining of the femoral arteries 14 days after collar placement in bone marrow recipients (Figure 4A). In addition, no β -galactosidase-positive cells were found within the neointima and media of the arteries. We only demonstrated X-gal positivity in the adventitia and perivascular space, the area between the adventitia and the position of the collar, of wild-type mice transplanted with bone marrow of Rosa26LacZ donors (Figure 4B).

Both macrophages and CD34-positive endothelial progenitor cells are derived from the bone marrow. The lack of X-gal positivity in the neointima and media suggested the absence of both cell-types in this region. Immunohistochemistry confirmed that macrophages (Figure 4C-D) and endothelial progenitor cells (Figure 4E-F) homed into the adventitia, but not towards the neointima and media of the collared femoral arteries.

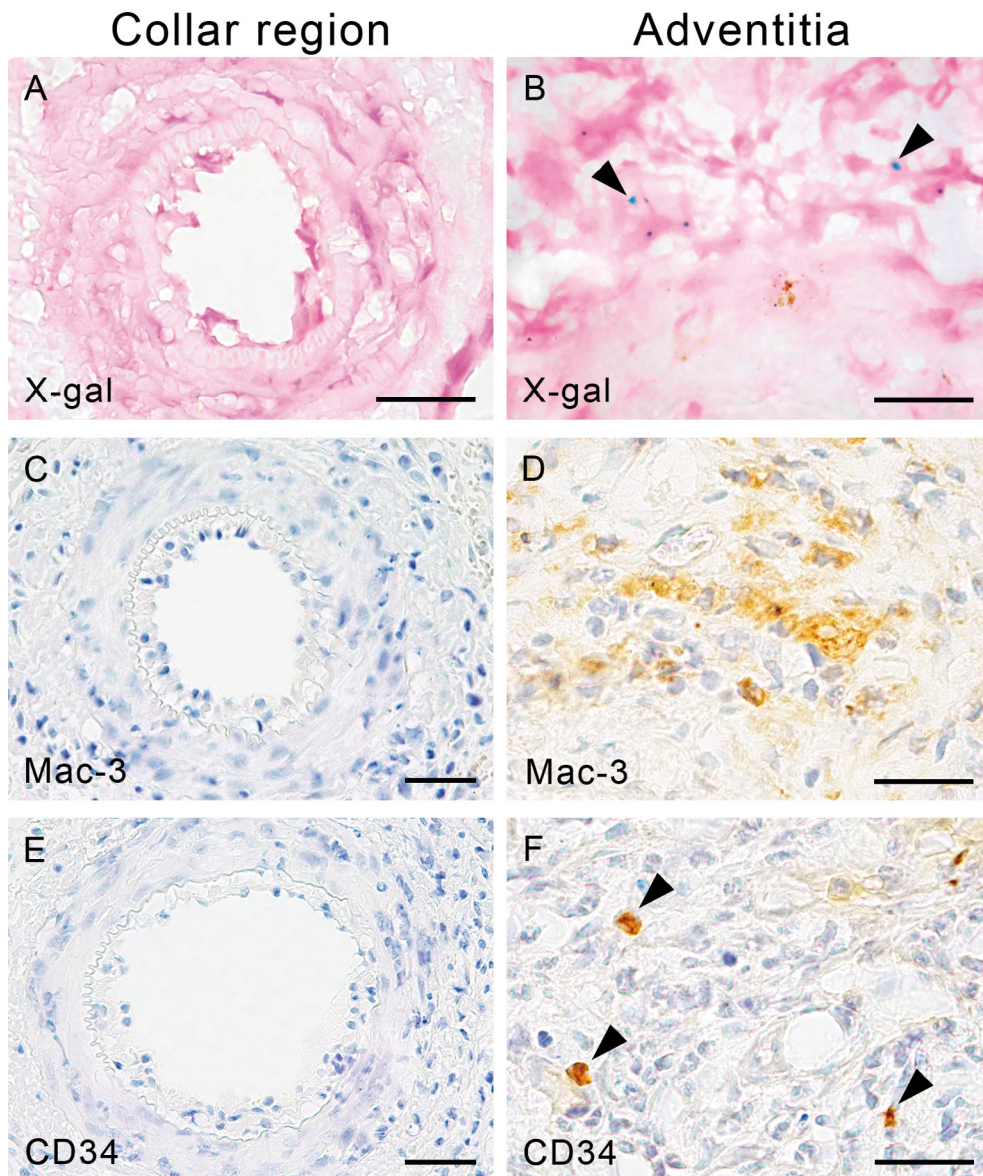


Figure 4. Bone marrow-derived cells in the adventitia. Representative cross-sections of the (A,C,E) intima and media and (B,D,F) adventitia 2 weeks after collar placement. (A-B) Beta-galactosidase expression, (C-D) presence of macrophages and (E-F) CD34-positive cells. Scale bars: 20 μ m.

The Effects of Collar Placement on Tgf- β Signal Transduction

Since Tgf- β signaling is important in EMT, we examined the consequences of collar placement on Tgf- β signaling in the initiation phase of EMT, 3 days after collar placement. Intense nuclear PSmad2 staining was detected in endothelial and smooth muscle cells of sham-operated femoral arteries (Figure 5A). In contrast, PSmad2 staining was markedly decreased and even absent in numerous areas within collared femoral arteries (Figure 5B). Nuclear expression of PSmad1/5/8 was present in the endothelial and smooth muscle cells of sham-operated, as well as collared femoral arteries (Figure 5C-D).

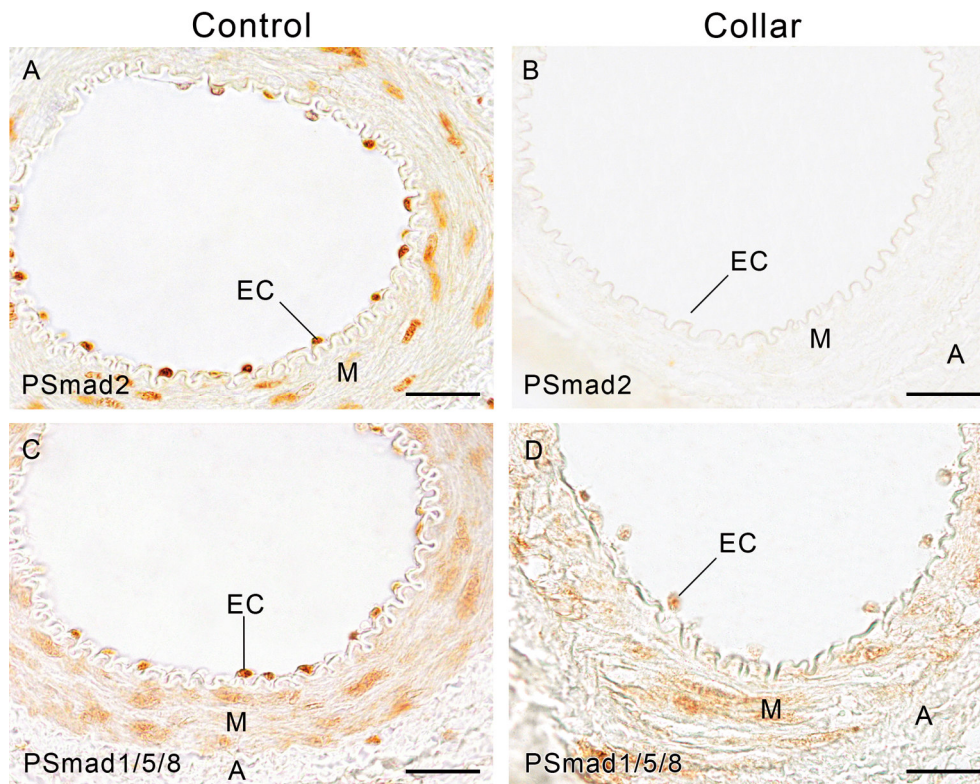


Figure 5. Tgf- β signaling pathway 3 days after collar placement. Representative cross-sections from (A,C) sham-operated and (B,D) collared femoral arteries were stained with antibodies against (A-B) PSmad2 and (C-D) PSmad1/5/8. EC indicates endothelial cell; M, media; A, adventitia. Scale bars: 20 μ m.

Discussion

In the present study we show that endothelial cells in the vasculature of adult mice still have the capacity to undergo EMT. By doing so, endothelial cells contributed to the total pool of mesenchymal cells that composed the neointima. Recently, it has been reported that adaptive intimal thickening may be important in the development of early stage atherosclerosis as it was found that it preceded pathological plaque formation.²² Furthermore, an important role has been ascribed to EMT in the process of cardiac fibrosis.²³ Constriction of the murine ascending aorta induced cardiac fibrosis and, in addition, evoked transdifferentiation of endothelial cells towards a fibroblast phenotype. Together, these data indicate that EMT could be important in development and progression of cardiovascular disease.

The early response to collar placement, at 3 days, was characterized by endothelial cell detachment and proliferation. Endothelial cell detachment is part of the process of cell scattering.²⁴ During cell scattering cell-cell adhesion complexes dissociate and the cells acquire cell motility. This process is a prerequisite for the occurrence of EMT. In addition to endothelial cell detachment, endothelial cell proliferation was observed. Smooth muscle cell proliferation was not detected. Our data are in accordance with a study that demonstrated that nonconstrictive collar placement solely induces intimal proliferation and has not adverse effects on the smooth muscle cell population of the media up to 2 weeks after collar placement.⁹ We conclude that perivascular collar placement around the femoral artery is a suitable technique to study EMT.

Our data indicate that the initial population of mesenchymal cells is of endothelial origin. Besides positivity of the endothelial cells for β -galactosidase in the Tie2LacZ reporter mouse model, part of the bone marrow-derived cells can express Tie2. Bone marrow transplantations were performed to elucidate the role of circulating cells in our model and to exclude bone marrow origin of the mesenchymal cells. Bone marrow-derived cells were only detected in the adventitia. Our results are supported by the data of Xu and colleagues who in the same animal model showed that all bone marrow-derived cells were directed to the adventitia and none were found in the intima.¹⁰ Thus, the β -galactosidase-positive mesenchymal cells are most likely of endothelial origin.

The β -galactosidase positivity seen in the mesenchymal cells was much less intense than that found in the endothelial cells. We showed that β -galactosidase staining was only present in the cellular membrane whereas in endothelial cells additional positivity was found in the nuclear membrane and Golgi. With subsequent cell divisions, the β -galactosidase that was present in the cellular

membranes diminished. These data indicate that Tie2 transcription in the mesenchymal cells is downregulated. Consequently, these cells lose endothelial cell-specific characteristics.

Mesenchymal cell characteristics on the other hand, were acquired. From 7 days after collar placement onwards a number of mesenchymal cells showed double positivity for β -galactosidase and α -smooth muscle actin. Our data are supported by a number of reports describing that during the initial phase of neointima formation the lesions contained α -smooth muscle actin positive cells.^{8,9} Since transmission electron microscopy revealed that the internal elastic lamina remained intact up to 14 days, active contribution of medial smooth muscle cells to the neointima between 7 and 14 days after collar placement can be excluded.

LeClair and colleagues recently reported that normal mouse carotid arteries highly express the Tgf- β -related proteins PSmad1/5/8 and PSmad2/3 in the nuclei of the endothelial cells.²⁵ Our data in the femoral artery are in concordance with this observation. We found high levels of PSmad1/5/8, as well as PSmad2 in endothelial and smooth muscle cells. High expression of these Smad proteins appears to be related to a differentiated and quiescent state.¹⁴ Tgf- β signaling pathways are not only associated with cell differentiation and quiescence, but also with the induction of EMT (reviewed by Zavadil²⁶). Therefore, for in vitro experiments this cytokine has been generally used to initiate EMT.²⁻⁴ Our data show that collar placement results in downregulation of Tgf- β /Alk5/PSmad2 signaling in endothelial cells. As a consequence, the balance between PSmad2/3 and PSmad1/5/8 shifted towards the Tgf- β /Alk1/Eng/Smad1/5/8 signaling pathway. Since the latter is associated with increased proliferation and migration of cells,²⁷ we postulate that Tgf- β signaling plays an important role in EMT in vivo. Additional studies on Smad proteins, Endoglin and the receptors Alk1 and Alk5 are foreseen to determine their role and the overall influence of Tgf- β .

In conclusion, the present study has provided evidence that adult endothelial cells in vivo have the capacity to undergo EMT. Furthermore, through EMT endothelial cells provide a first population of mesenchymal cells. We postulate that EMT may play an important role in the development and progression of cardiovascular disease.

Acknowledgements

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Chapter 6

Maternal Transmission of Risk for Atherosclerosis

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Maternal Transmission of Risk for Atherosclerosis

Abstract

Purpose of review

In the last 20 years, an increasing amount of epidemiological and pathological evidence has become available illustrating the relationship between an adverse in utero environment and increased risk of vascular disease in the offspring. It is now generally accepted that epigenetic phenomena, such as either DNA methylation or chromatin modifications or both mediate the long-term memory and thus developmental programming of cells and tissues.

Recent findings

In utero, the placenta and fetus are exposed to the metabolic, antioxidant and pro-inflammatory and anti-inflammatory signals from the mother and will likely respond specifically. In the fetus, these responses may lead to permanent changes either in DNA methylation or chromatin modification or both and these changes may lead to increased atherosclerosis susceptibility in adulthood. However, the molecular mechanisms responsible for the translation of an adverse maternal environment into permanent epigenetic changes are poorly understood.

Summary

In this review, we briefly summarize the possible signals crossing the placental barrier and discuss the molecular mechanisms of epigenetic programming in the developing fetus leading to increased atherosclerosis susceptibility of the vessel wall.

Introduction

Embryonic and fetal development is a delicately regulated process. Multiple signaling pathways, including transcriptional and (post)translational interactions, have to be framed in exact spatiotemporal windows during development. A small deviation in one of these processes can easily affect cellular differentiation or cellular contribution to developing organs. Many genetic defects and teratogenic agents such as ionizing radiation, drugs and environmental chemicals, can harm normal development and result in dramatic congenital malformations and even embryonic lethality. This knowledge had led in the 1970s to a world wide restriction on irradiation and medicine application during pregnancy.

The placenta plays a crucial, intermediary role in providing the proper microenvironment that allows normal development of the embryo and fetus and that adjusts the metabolism of both mother and child. Disturbances in the critical maternal-fetal metabolic balance, however, affect fetal growth ranging from a low birth weight to macrosomia (too large for the gestational age). In 1989, the epidemiologists Barker and colleagues¹ elegantly demonstrated the association of low birth weight with an increased risk of cardiovascular disease in adult life. This led to the “fetal origins hypothesis” proposing that cardiovascular risk is at least partially determined by the maternal environment in utero. The fetal origins hypothesis states that adaptation to unfavorable aspects of the maternal environment is beneficial to the developing embryo in utero. However, when the adult environment differs from the fetal environment, these adaptations may lead to an increased risk for cardiovascular disease. A subsequent burst of epidemiological studies both in humans and animals have proven the relation of maternal undernutrition and malnutrition,² diabetes mellitus³ and hypercholesterolemia^{4,5} on the one hand with size at birth, atherogenic lipid profiles and on the other hand with increased susceptibility to atherosclerosis in later life.

The challenging question is how an adverse maternal environment can be translated into an increased atherosclerosis susceptibility of the offspring. Which maternal signals can cross the placenta and what mechanisms are involved to predispose the developing embryo or fetus? In this review, we focus on the recent evidence that indicates that either the establishment of normal cell and tissue-specific DNA methylation patterns or chromatin modification status from early embryonic to late fetal life or both can be influenced by maternal signals.

Fetal Undernutrition

Protein restriction during pregnancy is associated with a low birth weight and an increased risk of coronary heart disease (CHD) mortality in adult life. The placenta

plays a crucial role in modulating the effects of protein restriction on the fetus and the mother by balancing their nutritional interests. Recent publications of the Dutch Hunger Winter (1944-45) family study⁶ demonstrate that exposure to famine during gestation results in an increased incidence of glucose intolerance, obesity, CHD, atherogenic lipid profile, hypertension and a series of noncardiovascular diseases.² Interestingly, the effect on cardiovascular risk factors depends on the trimester during pregnancy in which the embryo is exposed to famine. Because the effects of protein restriction are most likely exerted through different mechanisms depending on the developmental stage, the time frame of exposure is essential. Exposure to famine in the first trimester results in an increased CHD risk, though birth weight is normal. This is presumably due to adequate nutrition and catch-up growth later on during pregnancy. Remarkably, the intima-media thickness of the vessel wall, a marker of CHD, is reduced in these offspring. However, this reduction in intima-media thickness does not decrease the risk of CHD at adult age. Experiments in rats⁷ also demonstrate a reduced aortic wall thickness and elastin content after maternal undernutrition. Thus, as protein restriction in early fetal development can result in normal birth weight and a reduced intima-media thickness, these factors have a limited predictive value. Other factors associated with increased risk of atherosclerosis have to be considered while operating during development and maintained into adulthood.

Feeding pregnant rats a protein-restricted diet results in measurable epigenetic changes such as hypomethylation of the peroxisome proliferator-activated receptor alpha and hepatic glucocorticoid receptor promoters in the offspring.^{8,9} Interestingly, the altered phenotype of the offspring and hypomethylation of the glucocorticoid receptor is prevented by addition of folic acid to the diet,¹⁰ a key intermediate in methylation reactions. Expression of the DNA methyltransferase (Dnmt)1 is reduced by protein restriction.^{11,12} These experiments link protein restriction to hypomethylation of specific promoters through reduction of Dnmt1. These experiments also suggest that the actual signal conferred by the protein-restricted diet is a relative deficiency in one-carbon metabolism. Pharmacological blockade of maternal glucocorticoid synthesis also prevents part of the phenotype that is hypertension. This suggests that increased corticosteroid levels, as a consequence of food restriction, may be the actual signal.¹³ It has been shown that binding of the glucocorticoid receptor complex to DNA is associated with local DNA demethylation and chromatin remodeling, subsequently resulting in a stronger glucocorticoid response.¹⁴ The binding of glucocorticoid receptor complexes to fetal DNA during developmental programming of DNA methylation status may thus account for persistent epigenetic changes. Deficiency in one-carbon metabolism, subsequent changes in Dnmt1 activity, increased

corticosteroid levels and successive transcription factor mediated changes in DNA methylation, converge on the same mechanism that increases the risk of atherosclerosis. They all may well contribute to epigenetic programming in response to a protein-restricted diet.

Alternatively, a mechanistic link between gestational growth restriction and increased cardiovascular diseases may be found in shortening of telomere length. Telomere shortening is found in atherosclerotic plaques and the media of the aorta.¹⁵ A rapid (post)natal weight gain after growth restriction in rats impairs the normal upregulation of the antioxidant defense capacity seen during normal slow increase of weight.¹⁶ An increased number of DNA single-strand breaks are detected resulting in enhanced telomere shortening during aging of the rats. These data indicate that adaptive pathways coping with oxidative stress are adjusted during early embryonic stages to the prevailing environmental circumstances. During rapid changes in food supply, these defense mechanisms are no longer adequate and result in a pro-atherogenic condition.

Maternal Hypercholesterolemia

Cholesterol is an essential component for adequate development of the fetus as it is an integral part of cell membranes, for example lipid rafts. In the third trimester of human pregnancy, when fetal growth rate is fast and fetal requirements are enhanced, the majority of mothers develop gestational hypercholesterolemia. Through changes in maternal cholesterol metabolism, nutrients are provided for pregnancy maintenance and fetal growth. Therefore, gestational hypercholesterolemia is generally considered to be a natural phenomenon not directly posing a risk for cardiovascular disease in both mother and progeny. In contrast, examination of human aortas from fetuses from overt hypercholesterolemic mothers reveals an increased number and size of atherosclerotic lesions compared with the aorta from fetuses from normocholesterolemic mothers.⁴ An extended study¹⁷ in neonates and young children demonstrates a persisting effect of maternal hypercholesterolemia in the offspring as a more rapid progression of atherosclerosis was detected, even when the offspring was normocholesterolemic. These results were substantiated and expanded in an apolipoprotein E (apoE) heterozygous knockout mouse model showing that maternal hypercholesterolemia is associated with endothelial damage in the fetal vasculature. The harmful effects persist until adult life and accelerate atherosclerotic lesion formation even in absence of a genetic susceptibility to cardiovascular disease.^{5,18} A remarkable finding in the heterozygous apoE-deficient mouse model⁵ was that, after birth, neither difference in morphology, in intima-media thickness nor in lipid profile could be demonstrated between the maternally

exposed and nonexposed groups. The exposed group, however, develops substantial intimal hyperplasia in response to reduced shear stress levels. The reduced or disturbed flow over the endothelium represents an additional pro-atherogenic condition for the vessel wall.¹⁹ This indicates that atherosclerosis susceptibility can be imprinted in fetuses with arteries that do not as yet manifest atherosclerotic disease. The question is which cell types involved in intimal hyperplasia, for example endothelial cells, smooth muscle cells, fibroblasts or circulating bone marrow derived cells, are affected during development.

The signals conferred from the hypercholesterolemic mother to the fetus include fatty acid composition, oxidative stress, inflammatory stress and adaptive immunity (reviewed by Palinski and colleagues²⁰). Recently, strong evidence for the transmission of oxidative stress to the fetus and a potential role for the placenta in modulating this signal has been obtained.²¹ Oxidative stress in the fetus most likely results not only in damage of the vessel wall, but also in an antioxidative stress response to counter this damage. The transcription factors modulating this response during a critical period of development may permanently alter the structure of the chromatin by preventing or enhancing DNA methylation status and chromatin modifications. Alternatively, oxidative stress has been proposed to alter the availability of cofactors such as S-[methyl-3H]-adenosyl-L-methionine to Dnmts and chromatin modifying enzymes.²² Either or both mechanisms could operate to translate oxidative stress to epigenetic changes.

The placenta functions as an efficient barrier to inflammatory cytokine transfer from the mother to the fetus.^{23,24} However, high levels of circulating cytokines in the mother will affect the placenta, which may subsequently affect inflammatory cytokine expression in the fetus. The challenge is to dissect the effects of the affected placenta on the fetus from the effects of the maternally increased oxidative stress directly on inflammatory markers in the fetus. Similar to oxidative stress, maternal inflammatory stress will affect pro-inflammatory and anti-inflammatory gene transcription in the fetus. If this occurs during a critical period of development, the epigenetic setting of the DNA may be permanently altered.

The strongest evidence for a role of the immune system in transmitting atherosclerosis susceptibility comes from maternal immunization studies. Immunization of pregnant mothers with oxidized LDL,²⁵ *Streptococcus pneumoniae*²⁶ or phosphorylcholine²⁷ decreases the extent of atherosclerosis in the offspring. Developmental programming of the immune system is poorly understood. It is clear that the mother's immune system is tolerant for the semi-allogeneic fetus, in which regulatory T cells play an important role.²⁸ Antibodies are actively transferred from the mother to the fetus and the suckling neonate. This maternal-derived antibody repertoire will affect immune responses of the neonate

and will play a role in the development and setting of the adaptive immune system extending into the adult offspring.

It is clear that epigenetic programming in the fetus in response to maternal hypercholesterolemia may occur through several signals and molecular mechanisms. Which of these signals and mechanisms are dominant will differ depending on the type of response that is being considered in the adult. Global gene expression analysis using microarrays of adult noncompromised arteries in the above discussed apoE heterozygous knockout mouse model,⁵ demonstrates upregulation of genes involved in immune pathways and fatty acid metabolism in the offspring from hypercholesterolemic mothers. This indicates that the signals from the adverse maternal environment are programmed in basic cellular processes of the fetal vascular cells. Furthermore, these are maintained throughout adulthood. Future studies have to prove which changes in DNA methylation are responsible for the increased atherosclerosis susceptibility.

Other Maternal Drivers of Increased Atherosclerosis in Offspring

Both maternal diabetes mellitus type 1 or 2 and gestational diabetes are characterized by macrosomia and obesity. Maternal diabetes results in an increased aortic intima and media thickness and left ventricular mass in the macrosomic newborns.³ Furthermore, the newborns have an increased risk of diabetes type 2 and obesity. There is no consensus on whether fetal lipid levels are augmented,³ unaffected,²⁹ or even reduced³⁰ by maternal diabetes. Although lipid profiles may remain unaffected in cord blood in offspring of diabetic mothers, a striking effect is exerted on inflammatory pathways involving ICAM-1 and IL-6 levels.^{29,31} Subclinical inflammation and altered vascular remodeling, detected in newborns of diabetic mothers, are likely involved in the increased risk of pathogenesis of atherosclerosis in adult life. Whether the inflammatory pathways are permanently altered by changes either in DNA methylation or chromatin modification patterns or both are still unknown and a challenge for future research.

Comparable effects are observed in offspring from mothers smoking during pregnancy. The detrimental effects of tobacco do not only affect the smokers themselves, but both direct and passive smoking during pregnancy is a major risk factor of reduced birth weight and increased atherosclerosis of the offspring. In children of mothers who smoked during pregnancy,³² significant lower plasma cholesterol levels were detected compared with nonexposed offspring even after adjustment for reduced birth weight. Although this is suggestive for a reduced

atherosclerotic risk of the exposed offspring, a significant increase in plasma cholesterol levels of 0.12 mmol/L per 10 years was seen when compared with nonexposed children. This resulted in cholesterol levels exceeding those of nonexposed counterparts in adolescence. At adult age, the exposed group has an increased atherosclerotic risk compared with the nonexposed offspring. The combination of an increased aortic intima media thickness in the neonate³³ and the long-term adverse effects on offspring lipid metabolism in the exposed group may manifest accelerated atherosclerosis in adult life.

Conclusion

The correlation between exposure to an adverse maternal environment during development and increased risk for adult vascular lesion formation is well described in human epidemiological and human and mouse pathological studies. Increasing evidence from animal studies indicates that permanent changes either in DNA methylation or chromatin modification status or both are responsible for the epigenetic programming of increased atherosclerosis susceptibility. Changes in the normal programming of either DNA methylation or chromatin modification patterns or both during either sensitive embryonic or fetal developmental periods or both may be caused by presence or absence of DNA-transcription factor complexes on the substrate DNA, and/or changes in the activity of enzymes involved in DNA methylation and/or chromatin modification. Which mechanism is dominant will likely depend on the maternal trigger and signal. Developmentally programmed cell and tissue-specific patterns of DNA methylation are largely maintained throughout life and are scarcely altered during aging.³⁴ Thus, genome-wide methylation arrays compared with RNA expression profiles of exposed and nonexposed human specimens may provide an insight in the link between developmental programming and pathology. These changes in specific DNA methylation profiles may be better indicators of atherosclerosis susceptibility in the offspring as compared with for example birth weight and intima media thickness.

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Chapter 7

Altered Global Histone Methylation Profiles in Vascular Endothelial and Smooth Muscle Cells in Offspring Exposed to Maternal Apolipoprotein E-Deficiency

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Altered Global Histone Methylation Profiles in Vascular Endothelial and Smooth Muscle Cells in Offspring Exposed to Maternal Apolipoprotein E-Deficiency

Abstract

We recently demonstrated that atherosclerosis susceptibility of adult heterozygous apolipoprotein E-deficient ($\text{apoE}^{+/-}$) offspring from $\text{apoE}^{-/-}$ mothers is significantly increased as compared to adult $\text{apoE}^{+/-}$ offspring from wild-type mothers. Here, we investigated the hypothesis that the adverse maternal environment in $\text{apoE}^{-/-}$ mothers alters the epigenetic modifications of DNA and histones in the vasculature of the offspring and thereby programs atherosclerosis susceptibility. Differential methylation hybridization microarray analysis was performed to study methylation patterns of CpG islands in DNA from the aortic arch and carotid arteries. An immunohistochemical approach was used to detect global histone methylation modifications. The microarray revealed a trend in hypermethylation of several CpG islands in the $\text{apoE}^{+/-}$ mice from $\text{apoE}^{-/-}$ mothers compared with those from wild-type mothers. Profiles of histone triple-methylation modifications of lysines and the accompanying lysine methyltransferases on the other hand, were clearly differentially affected by the maternal apoE status in the vascular endothelial and smooth muscle cells. We conclude that chromatin modification marks in the vasculature were affected by maternal apoE-deficiency. The observed changes in global histone methylation modifications within the vascular cells are associated with altered atherosclerosis susceptibility of the offspring and could explain this epigenetic phenomenon.

Introduction

An increasing amount of epidemiologic and pathological evidence is provided that an adverse maternal environment during embryonic development is correlated with an increased risk of cardiovascular disease in the offspring in adulthood.^{1,2} The “fetal origins hypothesis” postulated by Barker² proposes that adaptation to an adverse maternal environment is favorable to the developing embryo. However, when the adult surroundings differ from the fetal setting, these fetal adaptations may lead to an increased disease risk.

In a recent animal study, we were able to demonstrate that maternal hypercholesterolemia and associated risk factors caused by apolipoprotein E-deficiency (apoE^{-/-}) induced a susceptibility for neointima formation in the offspring. Placement of a constrictive collar around the left carotid artery of adult apoE^{+/-} offspring from apoE^{-/-} mothers, resulted in extensive intima formation, both in the presence³ and absence (unpublished data) of high-cholesterol feeding. ApoE^{+/-} offspring from wild-type mothers were mainly nonresponders. These data indicate that atherosclerosis susceptibility may be programmed within the differentiating arteries during embryonic and fetal development and can persist into adulthood. The mechanisms underlying the observed atherosclerosis susceptibility are at present unknown. Global gene expression analysis on noncompromised (no collar) carotid arteries of adult apoE^{+/-} mice from apoE^{-/-} mothers, revealed upregulation of pathways involved in the immune response, fatty acid metabolism, and carbohydrate metabolism compared to offspring from wild-type mothers.³ Because the offspring are 100% genetically homogeneous, the observed differential expression patterns are likely to be governed by epigenetic mechanisms.

Epigenetic mechanisms affect gene expression by modification of the chromatin organization without changing the nucleotide sequence within a cell and ensures the maintenance of such an organization through mitotic cell divisions into the daughter cells. DNA methylation and covalent post-translational histone modifications are key players in the epigenetic regulation of the transcription machinery. Methylation of CpG sites within the DNA regulates gene expression, but is also thought to play an important role in silencing (retro)viral DNA sequences.^{4,5} As early as at the time of and directly after fertilization DNA demethylation of the paternal genome occurs, followed by de novo methylation. Both are important in the incorporation process of the maternal and paternal genomes.^{6,7} The mechanisms that apply or maintain DNA methylation are still not fully understood but involve many different proteins, and modified histones, and non-histone proteins such as DNA methyltransferases (Dnmts) like Dnmt1, and Dnmt3a/3b.^{8,9} In mammals, DNA methylation predominantly occurs symmetrically at a CpG dinucleotide, often clustered in so called CpG islands.¹⁰

The major targets for histone modifications are conserved residues located mainly in the amino-terminal tails of histones H3 and H4. These post-translational modifications include methylation, acetylation, phosphorylation, ubiquitination and sumoylation and together they establish the “histone code”. Histone methylation is thought to be the most stable of the modifications (reviewed by Shilatifard¹¹). Histones are methylated either on the lysine and/or arginine residues. These modifications affect gene transcription. Triple-methylation of lysine 27 in histone H3 (3Me-K27-H3) by the accompanying lysine methyltransferase EZH2 complex is an epigenetic mark generally associated with gene silencing. Until recently, triple-methylation of lysine 9 within histone H3 (3Me-K9-H3) by the lysine methyltransferase SUV39H1 was also thought to be a unique hallmark of gene silencing. However, Vakoc and colleagues showed that 3Me-K9-H3 is also present in transcribed regions of active genes indicating that 3Me-K9-H3 has a dual role.¹² In contrast to 3Me-K9-H3 and 3Me-K27-H3, 3Me-K4-H3 is linked to active transcription. Acetylation of histones reduces the affinity between histones and DNA thereby mediating and enhancing transcription.^{13,14}

We hypothesized that adverse factors associated with maternal apoE-deficiency alter either the DNA methylation and/or histone methylation modification patterns or both in embryonic vascular endothelial cells (EC) and smooth muscle cells (SMC) and that these differences persist into adulthood. To study the effect of maternal apoE-deficiency on DNA methylation patterns in the vasculature of apoE^{+/-} offspring, a differential methylation hybridization microarray analysis was performed. An immunohistochemical approach was used to detect post-translational modifications in the histone amino-terminal tails within the vessel wall. Detection of changes in DNA methylation and histone modifications within the vascular cells may provide us with a first indication of an underlying epigenetic mechanism to explain the increased atherosclerosis susceptibility in offspring from apoE-deficient mothers.

Methods

Mice

The apoE^{-/-} and wild-type C57Bl/6J mice were purchased from Charles River Laboratories (import agency for Jackson Laboratories). The apoE^{-/-} and wild-type mice were crossbred to generate genetically identical apoE^{+/-} offspring (n=13 each group) from apoE^{-/-} as well as from wild-type mothers. From weaning onwards (age 4 weeks) the animals received a Western-type diet (1% cholesterol, Hope Farms). Diet and water were provided ad libitum. The Committee on Animal Welfare, Leiden University Medical Center, approved all animal experiments.

Tissue Harvesting and Preparation

For the Differential Methylation Hybridization (DMH) Microarray Analysis the aortic arch and carotid arteries were harvested at age 8 weeks (n=8 each group). The mice were anesthetized and the thorax opened.³ Pressure-perfusion (76 mm Hg) was performed through the cardiac left ventricle with phosphate buffered saline (PBS) for 5 minutes. The arteries were dissected and immediately snap frozen. For immunohistochemistry, the carotid arteries were obtained at 20 weeks and fixed for 6 hours in 4% paraformaldehyde in 0.1 mol/L sodium phosphate buffer (n=5 each group). After fixation, the tissues were dehydrated in graded ethanol and xylene, and paraffin embedded. Transverse 5-μm sections were cut and serially mounted.

DMH Microarray Analysis and Bisulfite Sequencing

Analysis of differential methylation using CpG island containing microarrays was done essentially as described by others.^{15,16} Detailed protocols for the generation of methylated amplicons and the hybridization procedure for the DMH assay are available on request at <http://www.lgtc.nl>. In short, high molecular DNA was isolated from the aortic arch and carotid arteries as previously described.¹⁷ An amount of 600 ng DNA was digested with the restriction enzyme MseI. 5' Overhangs were ligated with the linkers 5'- AGG CAA CTG TGC TAT CCG AGG GAT-3' and 5'- TAA TCC CTC GGA -3'. Fragments were then digested with the DNA methylation-sensitive restriction enzymes BstUI and HpaII (Fermentas), followed by amplification. Fragments were labeled with CY3 or CY5 (GE Healthcare) fluorescent labels. Samples were hybridized on a single-spotted array containing 4.6 k mouse CpG-island clones (University of Toronto, Ontario, Canada: (<http://www.microarrays.ca/products>) from a library obtained from the Sanger Institute. Microarrays were hybridized conform a dye-swap procedure;¹⁵ a set of two samples was hybridized twice, switching the fluorescent labels. Microarrays

were scanned with the G2565BA microarray scanner of Agilent. To analyze the microarray data (two separate images), the circular features mode of GenePix 4.0 was used. Normalization of the data and analysis of differential methylation between the vasculature of apoE^{+/-} mice from apoE^{+/+} mothers and those from apoE^{-/-} mothers was performed in Rosetta Resolver software (<http://www.rosettabio.com>) by means of *t*-tests. Multiple test correction implied Bonferroni and Benjamini-Hochberg. Because none of the analyzed clones showed significant differences (corrected for multiple testing (MT)) in methylation between groups, the top 88 of the uncorrected MT potential differentially methylated clones with a *P*-value < 0.0001, sufficient intensities on the array, and a fold change of at least 1.2 were searched for nearby genes at: <http://data.microarrays.ca/cpghouse>. Clones which represented biologically relevant genes were validated by means of bisulfite sequencing.

Bisulfite Sequencing

Validation of potentially biologically relevant differentially methylated regions or CpG islands of nearby genes was performed by bisulfite sequencing as previously described.^{18,19} Approximately 500 ng of genomic DNA was used in the modifying bisulfite reaction. The following CpG island loci, in or near the respective gene, were selected for validation of the CpG microarray analysis: Prostaglandin-endoperoxide synthase 1 (*Ptgs 1*), Tubulin beta 2 (*Tubb2b*), Tumor necrosis factor receptor superfamily 4 (*Tnfrsf4*), Interleukin 12b (*IL12b*), GATA binding protein 2 (*Gata2*) and Cystin 1 (*Cys1*). Sequencing analysis was performed with Big Dye terminator kit (v3.1) of Applied Biosystems and analyzed on an ABI3100. Details on bisulfite PCR and sequencing primers are available on request.

The analyses revealed all loci to be completely unmethylated. To exclude a bias due to preferential amplification and to verify that the bisulfite PCR could detect unmethylated as well as methylated DNA, PCR-amplified (unmethylated) DNA was enzymatically methylated. In vitro methylation of extended PCR fragments of IL12b was performed by SssI CpG methylase (NEB, approximately 450 bp, primers available on request). The fragments were modified with the bisulfite reaction and checked for 100% methylation by HpaII digestion and after that diluted 1:1 with unmethylated PCR fragments. In this way, we generated a positive control of 50% methylated PCR *IL12b* fragments, which we amplified with our IL12b methylation detecting PCR assay. We found no evidence for preferential amplification or detection bias of our IL12b assay (data not shown).

Immunohistochemistry

Unless indicated otherwise the immunohistochemistry was performed as described earlier.^{3,20} Overnight incubation at room temperature was performed with rabbit anti-trimethyl-histone H3 (Lys 27) (1:1000, Upstate, Lake Placid, USA, Cat no. 07-449) and the accompanying lysine methyltransferase mouse anti-EZH2 (1:250, BD Biosciences, Breda, The Netherlands, Cat no. 612267), rabbit anti-trimethyl-histone H3 (Lys 9) (1:1000, Upstate, Cat no. 07-442) and its accompanying lysine methyltransferase rabbit anti-SUV39H1 (1:1000, Abcam, Cambridge, UK, ab38637), rabbit anti-trimethyl-histone H3 (Lys4) (1:1000, Upstate, Cat no. 07-473) and its accompanying lysine methyltransferase rabbit anti-hSet1 (1:500, Bethyl laboratories, Montgomery, USA, Cat no. IHC-00171), rabbit anti-acetyl-histone H3 (1:1500, Upstate, Cat no. 06-599), and rabbit anti-acetyl-histone H4 (1:2000, Upstate, Cat no. 06-866). Goat anti-rabbit biotin conjugate (1:200, Vector, Amsterdam, The Netherlands) with normal goat serum diluted in PBS was used as secondary antibody. For EZH2, the primary antibody was coupled overnight to polyclonal rabbit anti-mouse peroxidase conjugate (1:200, DAKO, Glostrup, Denmark) secondary antibody.²¹ Sections were incubated for 2 hours at room temperature. Biotin labeling was followed by incubation with Vectastain ABC (Vector). The SUV39H1 signal was enhanced with a CSA kit (DAKO, Glostrup, Denmark). 3-3' diaminobenzidine tetrahydrochloride (DAB) was used for visualization and counterstaining was performed with Mayer's hematoxylin.

Data Analysis

In randomly selected sections (5-10 each carotid artery) the number of positive and negatively stained EC and SMC were counted for each antibody. The data were analyzed with linear mixed model statistics. With this technique we were able to take into account the possible variation between individual mice. The differences were considered to be significant if $P < 0.05$ and the power $\geq 80\%$.

Results

DNA Methylation

The vascular material of a total of 16 mice, 8 apoE^{+/-} offspring from wild-type mothers and 8 apoE^{+/-} offspring from apoE^{-/-} mothers, were hybridized (dye-swap) on 16 CpG island microarrays. Analysis of differential methylation showed 88 regions with a significant difference (hypermethylation) in methylation pattern (for P -value ≤ 0.01 , P -value plot and table see Appendix). After Bonferroni and Benjamini-Hochberg correction for multiple testing no significant differences in methylation patterns between both groups of apoE^{+/-} mice were detected. We evaluated the 88 clones on intensity (on both dye-swap microarrays) and fold change. Furthermore, this statistical selection was submitted to the UHN mouse CpG island database (see methods) and evaluated for biological relevance with respect to the vascular phenotype difference between the two groups. The submitted clones were screened for being in the vicinity or within potentially relevant genes. This selection procedure yielded six promising loci: The genes *Ptgs1*, *Tubb2b*, *Tnfrsf4*, *IL12b*, *Gata2* and *Cys1*. However, bisulfite sequencing did not indicate that the CpG islands near/within *Ptgs1*, *Tubb2b*, *Tnfrsf4*, *IL12b*, *Gata2* and *Cys1* were differentially methylated.

Histone Modifications

3Me-K27-H3 and EZH2

The majority of EC nuclei were stained with anti-3Me-K27-H3 (Figure 1A-B). A trend towards an increase in the number of cells staining with the anti-3Me-K27-H3 antibody was observed in the carotid arteries of apoE^{+/-} offspring from apoE^{-/-} mothers compared with those of wild-type mothers (Figure 1C). The accompanying histone methyltransferase EZH2 (Figure 1E-F) colocalized and showed a significant enhancement of staining in the EC ($97.4 \pm 0.7\%$ versus $92.5 \pm 1.1\%$, $P = 0.026$, 1G). In SMC on the other hand, a significant decrease in the number of 3Me-K27-H3-positive SMC was detected in the carotid arteries of apoE^{+/-} offspring from apoE^{-/-} mothers relative to apoE^{+/-} offspring from wild-type mothers ($69.1 \pm 1.3\%$ versus $51.5 \pm 1.4\%$, $P = 0.005$, Figure 1D). EZH2 showed a similar tendency, although not resulting in statistical significance (Figure 1H). A striking finding was that not all SMC nuclei were stained completely. Partial nuclear staining was a common feature (clearly visualized in Figure 1A,F). This phenomenon of asymmetric nuclear staining was also seen in sections stained with the other antibodies.

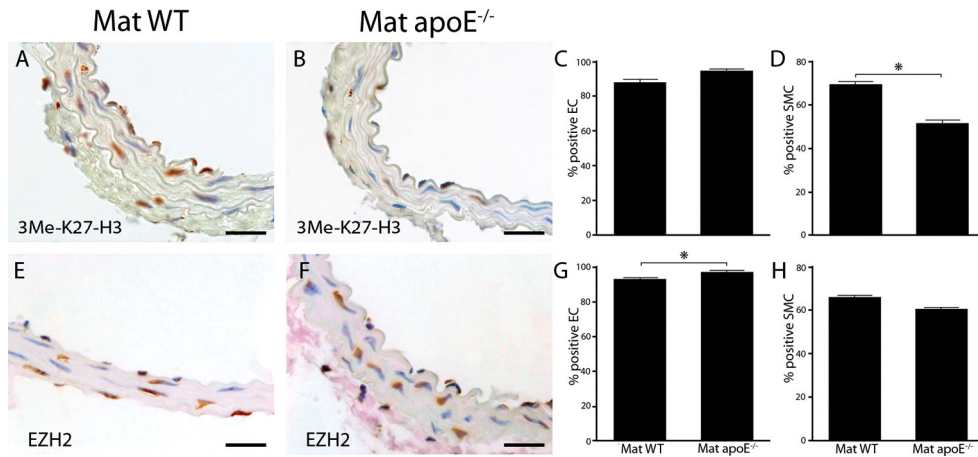


Figure 1. 3Me-K27-H3 and EZH2 staining in carotid arteries. Representative immunostaining for (A-B) 3Me-K27-H3 and (E-F) EZH2 in cross-sections from apoE^{+/+} offspring from wild-type mothers (Mat WT) and apoE^{-/-} mothers (Mat apoE^{-/-}). Scale bars: 20 μ m. Percentage of (C-D) 3Me-K27-H3-positive and (G-H) EZH2-positive EC and SMC in Mat WT and Mat apoE^{-/-} offspring. Note the clear and significant reduction in 3Me-K27-H3 staining in the SMC of Mat apoE^{-/-} offspring. In addition, Figure 1A and 1F clearly show partial nuclear staining in the SMC. Data are means $\pm$ SEM. Significant difference observed in (D) * P = 0.005 and (G) * P = 0.026.

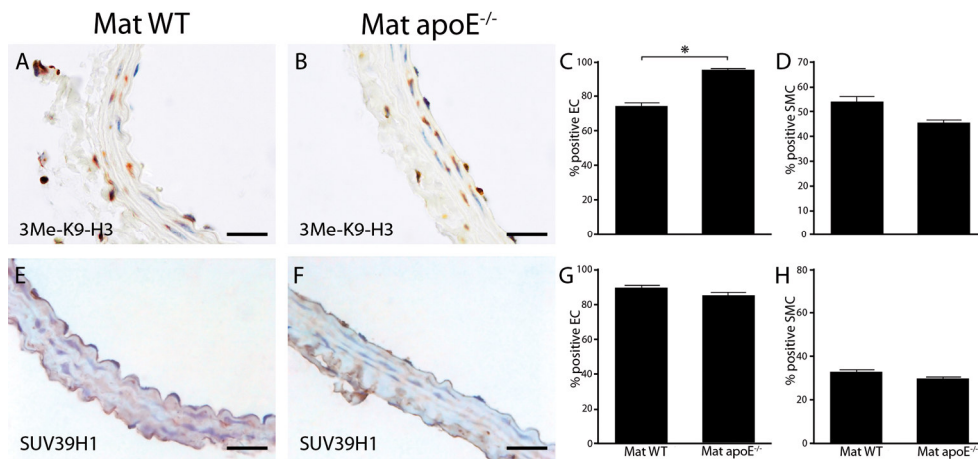


Figure 2. 3Me-K9-H3 and SUV39H1 staining in carotid arteries. Representative immunostaining for (A-B) 3Me-K9-H3 and (E-F) SUV39H1 in cross-sections from apoE^{+/+} offspring from wild-type mothers (Mat WT) and apoE^{-/-} mothers (Mat apoE^{-/-}). Scale bars: 20 μ m. Percentage of (C-D) 3Me-K9-H3-positive and (G-H) SUV39H1-positive EC and SMC in Mat WT and Mat apoE^{-/-} offspring. Note the evident and significant increase in 3Me-K9-H3 staining in the EC from Mat apoE^{-/-} mice. Data are means $\pm$ SEM. * P = 0.021

3Me-K9-H3 and SUV39H1

A striking significant increase in staining of 3Me-K9-H3 was detected in the EC of carotid arteries of apoE^{+/-} offspring from apoE^{-/-} mothers compared with those of wild-type mothers ($74.4 \pm 2.1\%$ versus $95.0 \pm 0.9\%$, $P = 0.021$, Figure 2A-C). The histone methyltransferase SUV39H1, which catalyzes the triple-methylation of K9-H3, colocalized with 3Me-K9-H3 in the EC (Figure 2E-G). No statistical differences were observed between SUV39H1-positivity in EC of apoE^{+/-} offspring from apoE^{-/-} mothers and apoE^{+/-} offspring from wild-type mothers. In contrast to the observed increase in EC of apoE^{+/-} from apoE^{-/-} mothers, a reduction in 3Me-K9-H3 and also SUV39H1 was observed in the SMC (Figure 2D,H). Remarkable was the low number of SUV39H1 positive SMC in all sections (29-33%).

3Me-K4-H3 and hSet1

In contrast to the gene repressive role attributed to 3Me-K27-H3 and 3Me-K9-H3, the marker 3Me-K4-H3 is linked to active transcription. No effects of maternal apoE-deficiency were detected in the vascular EC of all mice (Figure 3A-C, E-G). However, a significant reduction in the percentage of 3Me-K4-H3 positive SMC was seen in the media of apoE^{+/-} offspring from apoE^{-/-} mothers compared with apoE^{+/-} offspring from wild-type mothers ($59.4 \pm 1.6\%$ versus $42.8 \pm 1.7\%$, $P < 0.000$, Figure 3D). In addition, the accompanying lysine methyltransferase hSet1 revealed a significant decrease in staining ($36.1 \pm 1.0\%$ versus $25.0 \pm 1.0\%$, $P = 0.005$, Figure 3H).

Acetylation markers Ac-H3 and Ac-H4

Acetylated histones are normally associated with active transcription. Although histone H3 in EC and SMC nuclei appeared to be sensitive to alterations in methylation as a result of maternal apoE-deficiency, no significant differences were detected in the acetylation pattern of histone H3 (Figure 4A-C). The acetylation of histone H4 on the other hand, was significantly upregulated in EC of apoE^{+/-} offspring from apoE^{-/-} mothers compared with apoE^{+/-} offspring from wild-type mothers ($78.7 \pm 4.4\%$ versus $86.9 \pm 1.2\%$, $P = 0.015$, Figure 4E-G). Similar to the staining profiles observed with the methylation markers, also acetylation of both histone H3 and H4 appeared to be decreased in the SMC (Figure 4D,H).

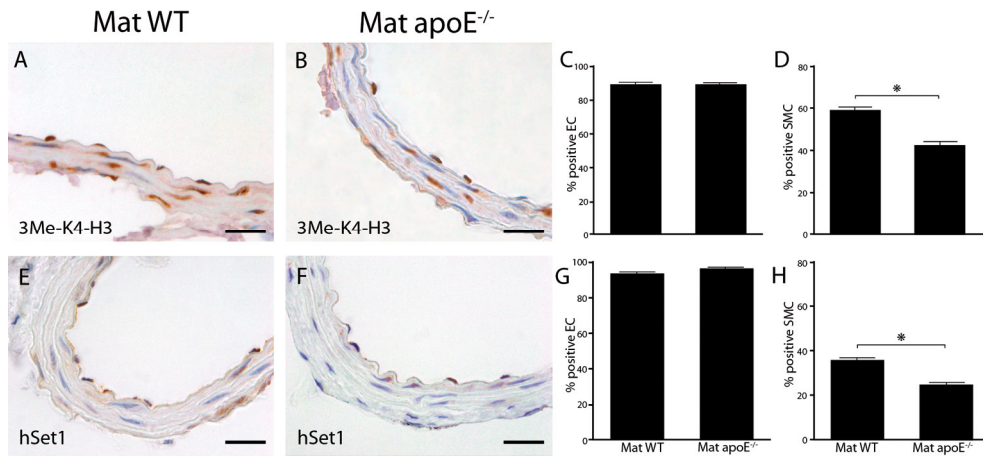


Figure 3. 3Me-K4-H3 and hSet1 staining in carotid arteries. Representative immunostaining for (A-B) 3Me-K4-H3 and (E-F) hSet1 in cross-sections from apoE^{-/-} offspring from wild-type mothers (Mat WT) and apoE^{-/-} mothers (Mat apoE^{-/-}). Scale bars: 20 μ m. Percentage of (C-D) 3Me-K4-H3-positive and (G-H) hSet1-positive EC and SMC in Mat WT and Mat apoE^{-/-} offspring. Note the significant reduction in 3Me-K4-H3 staining, as well as in the accompanying lysine methyltransferase hSet1 in the SMC from Mat apoE^{-/-} offspring. Data are means $\pm$ SEM. Significant difference observed in (D) * P < 0.000 and (H) * P = 0.005.

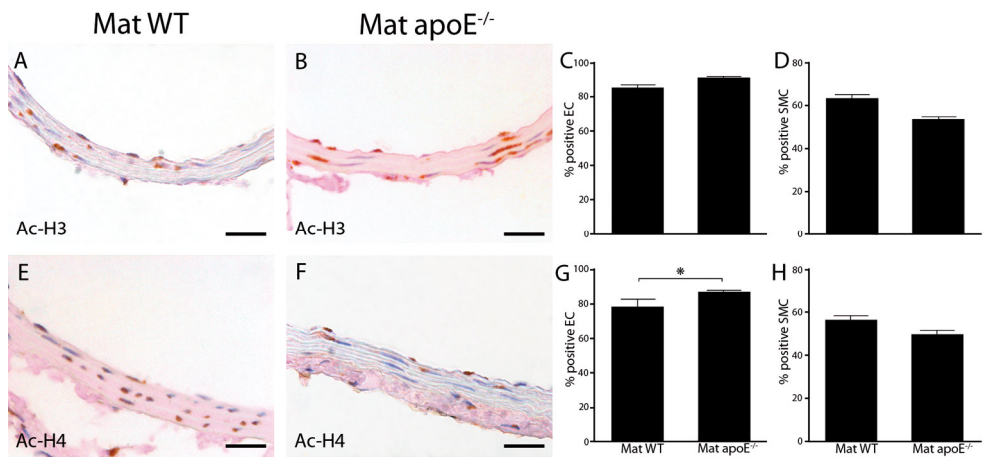


Figure 4. Ac-H3 and Ac-H4 staining in carotid arteries. Representative immunostaining for (A-B) Ac-H3 and (E-F) Ac-H4 in cross-sections from apoE^{-/-} offspring from wild-type mothers (Mat WT) and apoE^{-/-} mothers (Mat apoE^{-/-}). Scale bars: 20 μ m. Percentage of (C-D) Ac-H3-positive and (G-H) Ac-H4-positive EC and SMC in Mat WT and Mat apoE^{-/-} offspring. Note the significant increase in Ac-H4 positivity in EC from Mat apoE^{-/-} offspring. Data are means $\pm$ SEM. * P = 0.015.

Discussion

This study provides a first indication that epigenetic programming could be responsible for the long-term persistence of the effects of prenatal exposure to atherosclerotic risk factors. Tissue-specific alterations were detected in histone modification patterns in EC and SMC of apoE^{+/-} mice from apoE^{-/-} mothers relative to apoE^{+/-} offspring from wild-type mothers. This shift in histone modification patterns may explain the altered susceptibility for cardiovascular disease that may be acquired in utero and maintained throughout life.

The examination of global histone modifications, with emphasis on triple-methylation of lysine residues in histone H3, through an immunohistochemical approach provided us with information on differences in levels of global histone modifications and their accompanying lysine methyltransferases between individual cells and cell types. The distinction in the proportion of positive EC and SMC between carotid arteries of apoE^{+/-} offspring from apoE^{-/-} mothers and those from wild-type mothers was used to assess the level of modification in a tissue-specific way. A striking observation was the general increase of the number of positively stained EC in carotid arteries of apoE^{+/-} offspring from apoE^{-/-} mothers compared with apoE^{+/-} offspring from wild-type mothers for all used markers. In SMC on the other hand, an overall decrease was detected. The observed changes in histone modification profiles indicate differences in the activation status of the vascular wall. They are associated with exposure to the adverse maternal environment created by apoE-deficient mothers. It is not clear at the moment whether the altered profiles are the result of a direct effect of the microenvironment on the developing vessel wall in utero or a reflection of indirect influences of other affected or reprogrammed biological systems, as for instance the immune system. In both cases, the maternal apoE status is the cause of the observed histone modifications in the vessel wall of the adult offspring.

Few studies have been performed on the role of histone modifications in cardiovascular disease. It was demonstrated that athero-prone shear stress levels, an important risk factor of atherosclerosis characterized by low or oscillatory flow profiles, affected chromatin remodeling in cultured human SMC.²² Histone H4 acetylation was reduced in the promoter regions of α -smooth muscle actin and smooth muscle myosin heavy chain in cultured human SMC thereby promoting SMC dedifferentiation. In vivo, in low-density lipoprotein receptor-deficient mice histone acetylation was associated with pro-atherogenic genes and in regulation of the oxLDL receptor.²³ In contrast to the relative lack of data on the role of histone modifications in cardiovascular disease, histone modifications and their effects have already been extensively studied in cancer research. Many global changes in post-translational histone modifications have been found.

The presence of 3Me-K9-H3, 3Me-K27-H3 and EZH2 in promoter regions of tumor suppressor genes has been reported to induce vulnerability to aberrant *de novo* DNA hypermethylation in tumor suppressor genes.²⁴⁻²⁷ The global loss of acetylation at K16-H4 and trimethylation at K20-H4 has been associated with hypomethylation of DNA, an important feature of cancer cells.²⁸ Besides their function in carcinogenesis, changes in expression profiles of histone lysine methyltransferases have been linked to disease risk. Overexpression of EZH2 correlates with poor prognosis in various human cancer types.²⁹⁻³³ A particular combination of histone markers is predictive for risk of recurrence of prostate cancer and gastric adenocarcinomas.^{34,35} These data indicate that histone modifications play a key role in carcinogenesis and disease outcome. In our study we observed an increase in the number of cells with triple-methylation of K9-H3 and of K27-H3. Furthermore, we also noted an enrichment of EZH2 which catalyzes 3Me-K27-H3 in EC of apoE^{+/-} offspring from apoE^{-/-} mothers. In addition, 3Me-K27-H3 and 3Me-K4-H3 were significantly reduced in SMC. Together these alterations in global histone methylation modifications affect the cellular portrait of expressed genes ultimately providing an athero-prone environment.

No pathology was detected in the carotid arteries, as well as in the aortic arch of all apoE^{+/-} mice at age 20 weeks thereby indicating that the histone triple-methylation modifications preceded any sign of atherosclerosis.³ Challenging of the carotid arteries with postnatal risk factors for atherosclerosis, such as induction of athero-prone low shear stress through collar placement and high-cholesterol feeding, resulted in accelerated neointima formation in the apoE^{+/-} offspring from apoE^{-/-} mothers.³ In apoE^{+/-} offspring from wild-type mothers on the other hand, no lesions were found under the same conditions. These data indicate that in apoE^{+/-} mice from apoE^{-/-} mothers the vascular wall is primed towards a pro-atherogenic phenotype.

The DMH microarray analysis revealed trends of differentially methylated CpG islands in the vasculature of apoE^{+/-} offspring from apoE^{-/-} mothers compared with apoE^{+/-} offspring from wild-type mothers. The majority of these trends were set in the direction of hypermethylation of the CpG islands. We found no evidence of significant methylation differences when MT correction was applied. However, from these experiments we can not conclude that DNA methylation is not affected by maternal apoE-deficiency. When we take into account in terms of percentage the relatively small alterations in global histone methylation, DNA methylation most probably will also show subtle changes. From a technical point of view, the DMH microarray is probably not sensitive enough to pick up small changes. The DMH method is focused only on methylated regions, ignoring the possibility of the presence of a bulk unmethylated DNA; this bulk is washed away after MSeI

digestion. In that way it is feasible that a positive result is based on a minority of the total input material.

DNA methylation plays a role in the tissue-specific transcriptional regulation of genes. Nonetheless, we did not distinguish between EC and SMC in our search to try to detect alterations in CpG island methylation between the apoE^{+/-} offspring from apoE^{-/-} mothers compared with apoE^{+/-} offspring from wild-type mothers. With, for instance, laser dissection techniques EC could be separated from SMC. However, the yield of DNA of such small numbers of specific cells is far too low in order to detect differentially methylated regions by means of microarrays or bisulfite sequencing. Additional techniques will have to be used to determine the effect of maternal apoE-deficiency on DNA methylation in the vasculature of apoE^{+/-} offspring.³⁶

It is becoming clear that DNA methylation is intimately linked to histone modification patterns. Genes marked by 3Me-K27-H3 and the EZH2, a subunit of the polycomb repressive complex PRC2, may represent the main template for de novo DNA methylation in cancer.²⁴ In this regard, it has been suggested that EZH2 interacts with DNMTs thereby controlling DNA methylation.²⁶ Methylation of K9-H3 provides a binding platform for heterochromatin protein 1 to chromatin which subsequently facilitates recruitment of DNMTs.^{37,38} On the other hand, histone modifications have also been reported to precede alterations in DNA methylation and act independent of DNA methylation.^{24,39} The link between DNA methylation and post-translational histone modifications in our model remains to be established.

We realize that this study does not provide information on specific genes and their silencing, inactivation or activation as a result of exposure to maternal apoE-deficiency. Future research therefore is aimed at the identification in the different relevant cell types of the targets of the various histone methylation modifications investigated

In conclusion, epigenetic programming of susceptibility to adult cardiovascular disease is associated with the triple-methylation of several histones within vascular EC and SMC. These alterations may be acquired in utero and maintained throughout life and explain the increased atherosclerosis susceptibility of apoE^{+/-} offspring from apoE^{-/-} mothers as compared to apoE^{+/-} offspring from wild-type mothers.

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Appendix

Results

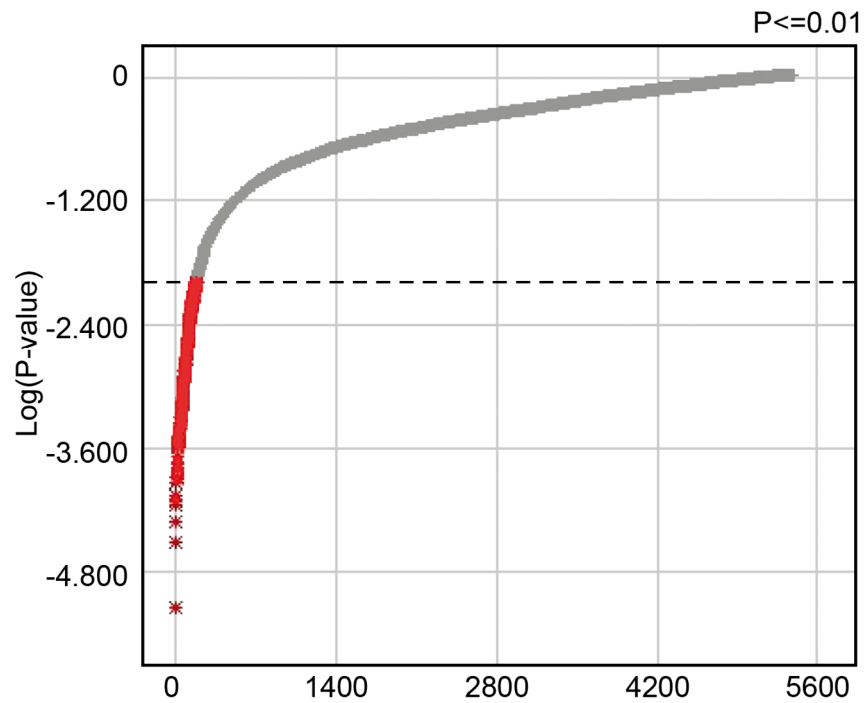


Figure 1. P-plot of 88 significant differentially methylated clones. Data based on student's *t*-test (not corrected for multiple testing) and presented as log *P*, red indicate $P \leq 0.01$.

Table 1. Significant different methylated clones (not corrected for multiple testing)

number	Sequence Name	Accession #	Fold Change	P-value	selected
1	MCPG2_50_G04	UHNmmcpG0012052	138,453	0,00000000030	Gata2
2	MCPG2_30_D12	UHNmmcpG0010104	163,494	0,00000020749	
3	MCPG2_72_E05	UHNmmcpG0014141	251,357	0,00000379349	
4	MCPG1_27_A02	UHNmmcpG0002313	193,665	0,00001000000	
5	MCPG1_64_G03	UHNmmcpG0005046	118,932	0,00001000000	
6	MCPG1_75_A12	UHNmmcpG0006489	245,650	0,00001000000	Gata2
7	MCPG2_66_G11	UHNmmcpG0013595	225,774	0,00001000000	
8	MCPG2_75_C02	UHNmmcpG0014402	149,212	0,00001000000	
9	MCPG1_27_G11	UHNmmcpG0002613	154,663	0,00002000000	
10	MCPG1_54_D12	UHNmmcpG0004943	145,658	0,00002000000	
11	MCPG1_57_G01	UHNmmcpG0004598	139,978	0,00002000000	Tnfrsf4
12	MCPG2_57_G12	UHNmmcpG0012732	118,405	0,00002000000	
13	MCPG2_74_C05	UHNmmcpG0014309	130,797	0,00002000000	
14	MCPG1_63_B03	UHNmmcpG0005051	114,430	0,00003000000	
15	MCPG2_24_B05	UHNmmcpG0009497	262,656	0,00003000000	
16	MCPG1_3_C01	UHNmmcpG0000006	168,124	0,00004000000	Ptgs1
17	MCPG2_54_A04	UHNmmcpG0012364	127,008	0,00004000000	
18	MCPG2_49_H11	UHNmmcpG0011975	116,785	0,00004451960	
19	MCPG1_34_G12	UHNmmcpG0003030	173,253	0,00005000000	
20	MCPG2_12_C11	UHNmmcpG0008363	235,166	0,00005000000	
21	MCPG2_66_E03	UHNmmcpG0013563	115,813	0,00005000000	Tnfrsf4
22	MCPG1_1_A01	UHNmmcpG0000001	268,587	0,00006000000	
23	MCPG2_31_D06	UHNmmcpG0010194	133,033	0,00007000000	
24	MCPG2_75_H04	UHNmmcpG0014464	176,242	0,00008000000	
25	MCPG2_77_A03	UHNmmcpG0014571	210,428	0,00012000000	
26	MCPG2_42_A12	UHNmmcpG0011220	254,723	0,00015000000	Ptgs1
27	MCPG2_60_E07	UHNmmcpG0012991	205,232	0,00018000000	
28	MCPG2_77_E12	UHNmmcpG0014628	245,634	0,00019000000	
29	MCPG2_47_G03	UHNmmcpG0011763	189,447	0,00021000000	
30	MCPG1_20_D12	UHNmmcpG0001887	187,978	0,00022000000	
31	MCPG1_28_C11	UHNmmcpG0002621	234,325	0,00022000000	Tnfrsf4
32	MCPG1_3_G11	UHNmmcpG0000334	260,267	0,00024000000	
33	MCPG1_31_F03	UHNmmcpG0002739	-113,372	0,00025000000	
34	MCPG2_69_F05	UHNmmcpG0013865	176,464	0,00025000000	
35	MCPG2_43_D03	UHNmmcpG0011343	-114,147	0,00027000000	
36	MCPG2_63_F10	UHNmmcpG0013294	129,488	0,00027000000	Tnfrsf4
37	MCPG2_24_B11	UHNmmcpG0009503	186,135	0,00028000000	
38	MCPG2_75_E11	UHNmmcpG0014435	208,235	0,00029070900	
39	MCPG2_75_E06	UHNmmcpG0014430	145,076	0,00043000000	
40	MCPG1_22_B09	UHNmmcpG0001788	221,737	0,00050000000	
41	MCPG1_3_F10	UHNmmcpG0000300	-116,111	0,00053000000	Tnfrsf4
42	MCPG1_26_C03	UHNmmcpG0001982	134,913	0,00059591800	
43	MCPG1_71_B08	UHNmmcpG0005979	-112,195	0,00067000000	
44	MCPG1_47_F07	UHNmmcpG0004020	264,273	0,00079000000	
45	MCPG1_60_B09	UHNmmcpG0004860	148,055	0,00079000000	
46	MCPG2_47_B07	UHNmmcpG0011707	134,492	0,00086000000	Tnfrsf4
47	MCPG2_62_E06	UHNmmcpG0013182	331,391	0,00089965600	
48	MCPG1_13_H02	UHNmmcpG0000832	139,470	0,00092000000	
49	MCPG2_18_C12	UHNmmcpG0008940	119,616	0,00092000000	
50	MCPG2_4_C02	UHNmmcpG0007586	212,782	0,00092000000	
51	MCPG2_69_D02	UHNmmcpG0013838	146,787	0,00097000000	Cys1
52	MCPG2_77_B10	UHNmmcpG0014590	226,755	0,00118000000	
53	MCPG2_39_B01	UHNmmcpG0010933	224,492	0,00124000000	
54	MCPG2_32_B01	UHNmmcpG0010261	-112,576	0,00134000000	

55	MCPG1_15_H07	UHNmmcpG0001362	134,237	0,00137000000	Tubb2b
56	MCPG2_66_D04	UHNmmcpG0013552	146,797	0,00141000000	
57	MCPG2_23_B3	UHNmmcpG0009399	183,980	0,00182000000	
58	MCPG2_29_G03	UHNmmcpG0010035	120,756	0,00184000000	
59	MCPG1_54_A07	UHNmmcpG0004777	140,258	0,00209000000	
60	MCPG2_24_A01	UHNmmcpG0009481	130,969	0,00221000000	
61	MCPG2_73_F10	UHNmmcpG0014254	125,207	0,00240000000	
62	MCPG1_51_E02	UHNmmcpG0004257	193,678	0,00261000000	
63	MCPG2_77_D11	UHNmmcpG0014615	171,896	0,00270000000	
64	MCPG2_54_E03	UHNmmcpG0012411	161,266	0,00304000000	
65	MCPG1_13_H07	UHNmmcpG0000992	125,019	0,00309895000	
66	MCPG1_78_B07	UHNmmcpG0006700	129,139	0,00310000000	
67	MCPG2_77_H04	UHNmmcpG0014656	205,780	0,00312000000	
68	MCPG2_76_G06	UHNmmcpG0014550	121,173	0,00365000000	
69	MCPG2_51_D03	UHNmmcpG0012111	-112,158	0,00417000000	
70	MCPG2_77_E08	UHNmmcpG0014624	233,735	0,00417000000	
71	MCPG1_27_E11	UHNmmcpG0002609	199,450	0,00433000000	
72	MCPG2_68_B06	UHNmmcpG0013722	-107,028	0,00437000000	
73	MCPG2_28_B11	UHNmmcpG0009887	-107,517	0,00469000000	
74	MCPG2_15_F02	UHNmmcpG0008678	-113,385	0,00538711000	
75	MCPG2_77_H10	UHNmmcpG0014662	241,198	0,00551000000	
76	MCPG2_77_E02	UHNmmcpG0014618	344,360	0,00567988000	
77	MCPG1_78_B01	UHNmmcpG0006508	180,482	0,00581000000	
78	MCPG2_77_B08	UHNmmcpG0014588	200,676	0,00654000000	
79	MCPG2_77_F01	UHNmmcpG0014629	187,156	0,00699000000	
80	MCPG2_19_G08	UHNmmcpG0009080	110,772	0,00721000000	
81	MCPG2_31_H07	UHNmmcpG0010243	-114,692	0,00796000000	
82	MCPG1_43_H09	UHNmmcpG0003704	-107,260	0,00800000000	
83	MCPG2_24_F06	UHNmmcpG0009546	164,537	0,00838000000	
84	MCPG2_77_E09	UHNmmcpG0014625	132,943	0,00883000000	
85	MCPG1_74_G05	UHNmmcpG0006261	125,285	0,00917000000	
86	MCPG2_24_G06	UHNmmcpG0009558	212,329	0,00942000000	
87	MCPG2_18_C09	UHNmmcpG0008937	183,268	0,00957000000	
88	MCPG2_4_E03	UHNmmcpG0007611	216,739	0,00969000000	

Chapter 8

General Discussion

Introduction

The aim of this thesis was to investigate the role of environmental prenatal risk factors for atherosclerosis on the susceptibility for cardiovascular disease in adult life. Using two mouse models of atherosclerosis, we show that intrauterine exposure to several of these factors results in prenatal programming and subsequent accelerated neointima formation in the offspring. The major conclusions and implications of our findings presented in this thesis will be discussed in this chapter.

Risk Factors of Intrauterine Programming of Adult Cardiovascular Disease

Maternal hypercholesterolemia

In this thesis we demonstrated in a murine model that maternal hypercholesterolemia most probably is not the most important trigger for intrauterine programming of atherosclerosis susceptibility and early manifestation of cardiovascular disease in the offspring. Profound maternal hypercholesterolemia did not induce fetal pathology in *Ldlr*^{+/-} offspring (Chapter 4). These data were substantiated by results from similar experiments performed in *apoE*^{+/-} mice (Chapter 2). Despite much lower cholesterol levels in *apoE*^{+/-} mothers compared with *Ldlr*^{+/-} mothers, we actually detected major endothelial damage in the vasculature of *apoE*^{+/-} fetuses. Together, these results indicate that other risk factors seem to be more important in the process of prenatal programming than solely maternal hypercholesterolemia.

Our data appear to conflict with the existing consensus that maternal hypercholesterolemia during pregnancy plays an important role in early stage atherosclerosis in the aortas of human and rabbit fetuses and neonates.^{1,2} However, it has to be taken into account that humans, as well as New Zealand White rabbits are genetically susceptible to atherosclerosis and fatty streaks could also be detected in these fetuses in absence of maternal hypercholesterolemia.^{1,2} *ApoE*^{+/-} and *Ldlr*^{+/-} mice on the other hand, are relatively athero-resistant. Human lipoprotein metabolism is characterized by a high LDL fraction and in rabbits the lipid metabolism shows comparable levels of LDL, VLDL and HDL.^{3,4} Wild-type mice on the other hand, are highly resistant to the development of atherosclerosis due to intrinsic low levels of total serum cholesterol, a high HDL fraction and very low levels of LDL.⁵⁻⁸ Only when fed a Western-type diet containing cholate⁹ or genetic manipulation, such as deletion of the *apoE* or *Ldlr* gene, mice will develop atherosclerotic lesions. Furthermore, differences in hemodynamic profiles between

humans, rabbits and mice may explain the discrepancy in intrinsic atherosclerosis susceptibility. Low shear stress is considered to be athero-prone^{10,11} and the average wall shear stress in human and rabbit carotid arteries is more than 6, respectively 3 times lower than in mice.¹² So, in contrast to all studies performed in humans and rabbits in which a genetic component may contribute to the phenotype, we most probably studied solely the effects of the environmental risk factor maternal hypercholesterolemia.

In case of genetic susceptibility to cardiovascular disease, presence of maternal hypercholesterolemia appears to accelerate and aggravate the phenotype.^{1,2,13} We confirmed this observation. We found intimal thickening in the fetal aorta of genetic susceptible *Ldlr*^{-/-} fetuses exposed to maternal hypercholesterolemia, but not in *Ldlr*^{-/-} fetuses from normcholesterolemic *Ldlr*^{+/-} mothers (Chapter 4).

As maternal hypercholesterolemia appears not to be the most important trigger for prenatal programming of atherosclerosis susceptibility, the consequences of hypercholesterolemia on programming probably are more subtle and comprise effects in regulation and modulation processes. For example, a recent study reported that intrauterine exposure to maternal hypercholesterolemia associated with *Ldlr*-deficiency affected the vascular responses to phenylephrine and Ca^{2+} in the carotid arteries of *Ldlr*^{+/-} and *Ldlr*^{-/-} offspring.¹⁴

Postnatal diet-induced hypercholesterolemia

Development of hypercholesterolemia in adult life is one of the major risk factors for cardiovascular disease. Therefore, we also investigated its contribution in manifestation of neointimal pathology in adult offspring. Our data showed that neointimal lesion initiation occurred independent of a high-cholesterol diet. Even without cholesterol feeding, neointimal lesions of considerable size developed in apoE^{+/-} offspring from apoE^{-/-} mothers (Chapter 3). A cholesterol-containing diet in these mice, however, severely accelerated and aggravated the phenotype (Chapter 2). Although diet-induced hypercholesterolemia is not the most important trigger for neointima manifestation in adult life, it exerts additional harmful effects on the development of cardiovascular disease.

Oxidative stress

Studies in rabbits in the early 1990s showed an association between hypercholesterolemia and oxidative stress.¹⁵⁻¹⁷ In addition, it has been reported that murine apoE-deficiency leads to high levels of reactive oxygen species (ROS) in the circulation and tissues¹⁷ and pregnancy itself¹⁸ is a condition that favors

oxidative stress. Therefore, the effects of maternal oxidative stress also needed to be taken into account in our studies.

In an initial attempt to detect oxidative stress in the fetus, we examined the presence of endproducts of glycation and lipid peroxidation, CML and MDA-LDL respectively, in the vascular wall. We did not find any sign of deposition of both proteins (unpublished data). However, from these data we can not conclude that maternal oxidative stress does not affect oxidative stress processes the offspring. The data are too preliminary and incomplete. Further studies on ROS detection and intermediate and endproducts of oxidation in the fetal circulation and vasculature have to be performed to gain more insight into this matter.

In addition to the formation of oxidation products, maternal oxidative stress may affect gene expression patterns in the offspring. Maternal oxidative stress could influence intrauterine programming in two ways. Maternal ROS may permeate the placenta and through peroxidative chain reactions in the fetal circulation affect signaling processes and induce a molecular memory, through for example DNA methylation.¹⁹⁻²¹ Alternatively, maternal reactive metabolites can trigger the fetal tissues for elevated ROS formation, thereby initiating a harmful intrinsic response leading to epigenetic changes. Little is known about the capacities of embryos and fetuses to form ROS. Presence of MDA-lysine and 4-HNE-lysine epitopes in fatty streaks in the human fetal aorta suggests that human fetuses themselves are capable of generating ROS.¹ Indirect measurements of oxidative stress in rat embryos/fetuses demonstrated that from gestational day 10 onwards oxygenation and dependence on oxidative metabolism increases.²² As early as gestational day 12-14 fetal rat tissues should have the capacity to form ROS.^{19,22}

Another factor we have to consider is that mitochondria, known to be major sites of ROS production and subsequent oxidative stress,²³ are maternally inherited.²⁴ As a result, the source of the mitochondria of apoE^{+/-} offspring from apoE^{-/-} mothers and apoE^{+/-} offspring from wild-type mothers differs. Microarray and gene set enrichment analysis on adult carotid arteries showed that several pathways associated with mitochondria were upregulated in the vasculature of adult apoE^{+/-} offspring from apoE^{-/-} mothers compared with apoE^{+/-} offspring from wild-type mothers (Chapter 2). Oxidative phosphorylation, the Krebs cycle, and carbohydrate and fatty acid metabolism were affected. Whether these functional changes can be explained by the origin of the mitochondria remains to be established.

It has to be mentioned that mitochondria themselves can also be compromised by severe and/or prolonged oxidative stress.²⁵⁻²⁸ Based on these

observations we suggest that mitochondrial function in the offspring may be influenced by extensive exposure to maternal oxidative stress.

Immune system

When we evaluate the results of our studies in the apoE^{+/-} and the Ldlr^{+/-} mouse models (Chapter 2-4), we can conclude that the effects of maternal apoE-deficiency on atherosclerosis susceptibility in the offspring are far more detrimental than those of maternal Ldlr-deficiency. The main functions of apoE and Ldlr involve lipid metabolism. The presence of apoE on VLDL, IDL, HDL and chylomicron particles facilitates rapid uptake from the circulation by high-affinity binding to the Ldlr and the Ldlr-related protein LRP. ApoE-deficiency results in accumulation of lipoproteins in the circulation because these particles cannot bind with high efficiency to either receptor.^{5,6} Ldlr-deficiency on the other hand, leads to impaired clearance of lipoproteins due to absence of one of the two receptors.^{29,30} Deficiency for either apoE or Ldlr evokes profound (diet-induced) hypercholesterolemia associated with oxidative stress.

Besides a role in cholesterol metabolism and transport, apoE also has anti-inflammatory properties,³¹ such as inhibition of expression of vascular cell adhesion molecules on endothelial cells,³² inhibition of lipid oxidation,³³ suppression of type I inflammatory responses³⁴ and regulation of clearance of apoptotic cells.³⁵ Lack of apoE as a consequence, adds an inflammatory component to the maternal phenotype.

Increased inflammation in pregnant apoE^{-/-} mothers during pregnancy most probably has harmful effects on the vasculature of the offspring. In apoE^{+/-} fetuses from apoE^{-/-} mothers severe damage to the endothelial cell lining was found whereas endothelial injury was neither seen in the vasculature of apoE^{+/-} fetuses from wild-type mothers (Chapter 2), nor in fetuses from immune competent Ldlr^{-/-} mothers (Chapter 4). In adult carotid arteries of apoE^{+/-} offspring from apoE^{-/-} mothers, which appeared normal at morphological examination, an upregulation of gene expression was found in genes involved in IL-12, IL-17 and T cell cytotoxicity pathways in comparison to apoE^{+/-} offspring from wild-type mothers (Chapter 2) suggesting a basic pro-inflammatory shift.

Maternal inflammation can adversely affect the fetal vasculature, but can also interfere with the construction of offspring immunity. A first indication of intrauterine programming of the immune system of the offspring was found when we compared intrinsic bone marrow composition between 8-weeks old apoE^{+/-} offspring from apoE^{-/-} and apoE^{+/-} offspring from wild-type mothers (unpublished data). Significant differences were found between both groups in bone marrow constitution, characterized by reduced numbers of granulocytes and monocytes.

The latter was associated with a phenotypic shift towards a more athero-prone phenotype, Ly6C^{hi}/CD31^{hi}. Differences in bone marrow composition and peripheral blood constitution between apoE^{+/-} offspring from apoE^{-/-} mothers and apoE^{+/-} offspring from wild-type mothers point toward an altered response in case innate or adaptive immunity is challenged. The role of programmed bone marrow in neointima formation remains to be established.

Several mechanisms can be proposed to explain the alterations observed in the immune system of apoE^{+/-} offspring from apoE^{-/-} mothers. Soluble factors, like cytokines in the maternal circulation, may pass the placenta and modulate fetal innate and adaptive immunity. Placental passage of maternal antigens could evoke autoimmune responses in the fetus. Furthermore, a number of studies have reported that maternal erythrocytes, platelets, granulocytes and lymphocytes, all cell types derived from the bone marrow, may pass the human placenta.^{36,37} They were detected in cord blood and in fetal blood samples obtained in the third trimester of pregnancy.³⁸ In mice, in late fetal stages lymphocyte-like maternal cells were found in the bone marrow of immune competent mice.^{39,40} Appearance of maternal cells in the bone marrow coincided with the start of hematopoiesis at these sites. These data suggest that maternal cells have a functional role in the foundation of the immune system of the offspring. Remarkably, even at 12 weeks after birth maternal cells could be detected in the offspring.⁴⁰ By exerting effects on the stromal microenvironment these cells could influence bone marrow composition and reactivity thereby contributing to the distinction in phenotype as observed between the apoE^{+/-} offspring from apoE^{-/-} mothers and those from wild-type mothers.

Intrauterine Programming of Adult Cardiovascular Disease

Post-translational histone modifications and DNA methylation

In this thesis we demonstrated that intrauterine exposure to maternal apoE-deficiency accelerated neointima formation in the offspring (Chapter 2,3). We conclude that the intrauterine environment in the apoE knockout mouse model poses a major risk for adult cardiovascular disease. Understanding of the underlying signals and epigenetic mechanisms through which prenatal programming is constituted is essential, especially for the development of prevention and treatment strategies. Recent literature governing these processes was reviewed in Chapter 6. Epigenetic memory is established by chromatin remodeling that can be initiated by among other things DNA methylation of CpG islands⁴¹ or post-translational modifications of the histone proteins of the nucleosome.⁴²

In theory, every individual cell of the embryo/fetus can be exposed to the adverse intrauterine environment created by maternal apoE-deficiency. As a result, all cells of the offspring may contribute to the founding of the atherosclerosis susceptibility as seen in apoE^{+/-} offspring from apoE^{-/-} mothers. Examination of the involvement of each tissue type is an almost impossible task. We decided to focus on cell types known to be important in atherosclerosis development.

The process of atherosclerosis starts with endothelial dysfunction.^{43,44} In addition, we demonstrated that endothelial cells themselves can contribute to the initial lesion development (Chapter 5). Adverse influences exerted by maternal apoE-deficiency on the vasculature of the offspring therefore may have major consequences on the cardiovascular disease risk.

Examination of adult carotid arteries of apoE^{+/-} offspring from wild-type mothers and apoE^{+/-} offspring from apoE^{-/-} mothers revealed clear tissue-specific effects of maternal apoE-deficiency on the global histone triple-methylation patterns (Chapter 7). In endothelial cells a general upregulation in triple-methylation and acetylation was observed whereas in smooth muscle cells an opposite response was found, namely an overall reduction in staining. The observed changes in histone modification profiles indicate differences in the activation status of the vascular wall. They represent alterations in chromatin structure and gene transcription control⁴² and are the resultant of a temporary exposure to the adverse maternal environment created by maternal apoE-deficiency. It is currently not clear whether the altered profiles are the result of a direct effect of the microenvironment on the developing vascular wall in utero or a reflection of indirect influences of other affected or reprogrammed biological systems, as for instance the immune system. In both cases the maternal apoE status is the cause of the observed histone modifications in the adult. The effect of maternal apoE-deficiency on DNA methylation in the vasculature of apoE^{+/-} offspring and the link between DNA methylation and post-translational histone modifications in our model remains to be established.

Prenatal programming by maternal apoE-deficiency can be induced between the time of conception and birth. During embryonic development multiple signaling pathways, including transcriptional and (post)translational interactions, have to be framed in exact spatiotemporal windows. A small deviation in one of these processes can influence cell lineage determination or cellular contribution to developing organs. Thus, timing and duration of exposure are of vast importance in establishing intrauterine programming. A recent study on the long-term consequences of prenatal exposure to famine as a result of the Dutch Hunger Winter (1944-1945) reported that very early mammalian development appears to be a crucial period for establishing and maintaining epigenetic marks.⁴⁵

Until now, our research focused on late fetal stages only (Chapter 2). Because all organs and cell types are founded at this time point, the sensitivity of a fetus to an adverse intrauterine environment may be lower than during the embryonic period. Examination of embryos and fetuses at different and foremost earlier time points in gestation could provide more information about the critical time windows and the course of development in presence of maternal apoE-deficiency. Furthermore, these studies may contribute to our understanding of the moment of the establishment of atherosclerosis susceptibility. Does an adverse intrauterine environment induce a memory that only manifests itself after being challenged by for example collar placement or an athero-prone diet? Or does it already during prenatal life result in changes in the regulation of many pathways and if so, can it be measured? Future efforts will focus on the specific genes that are affected by either DNA methylation and/or post-translational histone modifications to be able to answer above-mentioned questions.

Genomic imprinting

Although we assigned the atherosclerosis susceptibility as observed in apoE^{+/-} offspring from apoE^{-/-} mothers to the adverse intrauterine environment created by maternal apoE-deficiency, we can not exclude that all, or part, of the effects are caused by genomic imprinting. In genomic imprinting, specific genes or loci show different epigenetic patterns depending on the parent of origin.⁴⁶ In certain situations the inheritance of the maternal or paternal allele containing a mutation even determines the type of congenital disorder that will develop. For example, inheritance of the maternal chromosome 15 containing a partial deletion in the long-arm results in the development of the Angelman syndrome in the progeny, whereas transmission of the paternal allele containing this mutation leads to the Prader-Willi syndrome.⁴⁷

In a recent study it was estimated that approximately 600 loci in the murine genome are potentially imprinted.⁴⁸ If there exists a distinction in the epigenetic marks between the maternal and paternal apoE allele, and likely other loci as well, this could explain the distinction in atherosclerosis susceptibility between apoE^{+/-} offspring from apoE^{-/-} mothers and those from apoE^{-/-} fathers (and wild-type mothers). The role of genomic imprinting in the induction of atherosclerosis susceptibility can be studied by embryo transfer experiments in which early apoE^{+/-} blastocysts from wild-type mothers are transferred to apoE^{-/-} females and vice versa. If the apoE^{+/-} fetuses from wild-type mothers transplanted into apoE^{-/-} recipients show vascular injury at E17.5 similar to that described in Chapter 2, an inheritable effect of the apoE allele most likely can be excluded. In case no endothelial damage can be detected in this group and pathology is seen in the

vasculature of apoE^{+/-} blastocysts from apoE^{-/-} mothers transplanted to wild-type recipients, we can conclude that genomic imprinting is responsible for induction of atherosclerosis susceptibility in apoE^{+/-} offspring from apoE^{-/-} mothers. Should the embryo transfer experiments prove that genomic imprinting is important, transgenerational inheritance should also be investigated.^{49,50} This could be done by assessment of cardiovascular disease risk in the second generation of apoE^{+/-} progeny, obtained by intercrossing of the F₁ generation of apoE^{+/-} offspring.

Clinical Implications

In this thesis we have reported that intrauterine exposure to maternal apoE-deficiency, and to a lesser extent maternal Ldlr-deficiency, induces susceptibility for cardiovascular disease in mice that persists into adulthood. Even though the results are obtained in murine models of atherosclerosis, they can give rise, through interpretation and extrapolation of the data, to the development of treatment and prevention strategies in both pregnant women and offspring.

Prevention and treatment mothers

In fact, not many reports are available on the normal progress of, and fluctuations in, plasma cholesterol levels, and a degree of inflammation and oxidative stress markers during pregnancy, not to mention the consequences for the offspring. Plasma cholesterol levels during pregnancy increase between 25-50% and the majority of pregnant women develop gestational hypercholesterolemia.^{51,52} Changes in maternal cholesterol metabolism are generally considered to be a natural phenomenon not directly posing a risk for cardiovascular disease in both mother and progeny. Cholesterol is an essential component for adequate development of the fetus, as it is an integral part of cell membranes. It is, however, not known at which threshold the effects of gestational hypercholesterolemia switch from being beneficial to the embryo/fetus to detrimental. This not only accounts for hypercholesterolemia, but also for hypocholesterolemia during pregnancy, which is associated with preterm delivery and reduced birth weight.⁵³⁻⁵⁵

Since we demonstrated in this thesis that maternal hypercholesterolemia most probably is not the most important trigger for intrauterine programming of athero-susceptibility and manifestation of pathology, (Chapter 3,4) the role of apoE, inflammation and oxidative stress on intrauterine programming should be investigated. In a recent study, a weak association between elevated maternal C-reactive protein, an indicator of inflammation and oxidative stress, and atherosclerosis development in the offspring was found.⁵⁶ Examination of other markers of systemic inflammation, such as TNF α , IL-6, IL-7, IL-8, serum amyloid A,

fibrinogen, and markers of endothelial cell function like sCAMs, may shed more light on the role of inflammation and oxidative stress on intrauterine programming.⁵⁷⁻⁵⁹ Altogether, we would like to recommend the initiation of an epidemiological prospective follow-up study in which, besides routine assessment of plasma cholesterol levels, a search for, and identification of, inflammatory and oxidative stress markers will be performed. This will increase our understanding on metabolic changes during pregnancy and the balance between maternal production and fetal needs. In addition, general monitoring may identify a high-risk population of women for which prevention and treatment strategies can be developed that benefit both mother and child.

In gestational hypercholesterolemia, the most conventional treatment strategy is dietary intervention.⁶⁰ In spite of moderate cholesterol-lowering effects, the CARDIPP study showed that dietary intervention has limited effects on biomarkers for inflammation.⁶¹ Dietary intervention therefore appears not to be the most ideal method to reduce the maternal burden. Many lipid-lowering and anti-inflammatory drugs exist that are prescribed in case of hypercholesterolemia or other metabolic disorders. However, they can not be safely prescribed during pregnancy.⁶² Statins, the most potent drugs that can be prescribed for cholesterol-lowering are contraindicated during pregnancy. This is the result of limited and inconclusive experimental data. Studies on the incidence of congenital birth defects after use of statins in the first trimester of pregnancy revealed a limited numbers of abnormalities among live births.⁶³⁻⁶⁵ There is still debate about whether or not these abnormalities are the consequence of statin exposure. The involvement of statins in spontaneous abortions in early pregnancy has never been examined. Furthermore, resultant cholesterol lowering and other pleiotropic effects^{66,67} most probably exert more subtle effects than induction of congenital birth defects. These influences have not been identified thus far. Finally, we have to realize that cholesterol lowering may establish cholesterol levels below the threshold necessary to provide for the fetal needs thereby harming the unborn. The results of this thesis could be of interest for the pharmaceutical industry as we provide new perspectives on how intrauterine atherosclerotic risk factors possibly exert their effects (Chapter 6,7).

Many animal studies predicted a bright future for dietary or synthetic antioxidants. They not only affect LDL oxidation, but have many additional biological effects. Probucol for example has been reported to reduce atherosclerotic lesion formation in rabbits,⁶⁸ monkeys,⁶⁹ rats,⁷⁰ and hamsters.⁷¹ Only in mice, atherogenesis was stimulated after probucol treatment.^{72,73} Clinical trials revealed that next to LDL lowering, probucol also reduced HDL levels.⁷⁴ Subsequently, clinical use was discarded. Studies on vitamin E showed

inconsistent results in animals^{2,75-78} and clinical trials in human demonstrated no beneficial effects.⁷⁹⁻⁸¹ Beta-carotene supplementation even significantly increased cardiovascular disease risk in humans.⁸²⁻⁸⁴ Overall, antioxidant treatment strategies are not as effective as expected and use in pregnant women therefore should not be recommended.

In addition to all the effort that has been put in studies on statins and antioxidants, progress has been made in the development of treatment regimes that act via anti-inflammatory effects. A growing body of evidence suggests a role for inflammatory pathways in adverse reproductive outcomes.⁸⁵ The two main intervention strategies are immunosuppressive drugs and immunization by vaccines. As far as anti-inflammatory drugs are concerned, it is not known whether use has harmful effects on the mother and fetus. Future studies have to be performed to gain more insight. The development of many vaccines is still in the pre-clinical phase, but they seem very promising (reviewed by Riley and colleagues⁸⁶). In the setting of intrauterine programming of adult cardiovascular disease, Yamashita and colleagues performed maternal immunization studies with oxLDL and KLH before pregnancy.⁸⁷ Atherosclerosis in the offspring was significantly reduced in case of maternal immunization with oxLDL. Whether vaccines in the future can be prescribed to pregnant women, without adverse effects on the mother and fetus, remains to be established.

Prevention and treatment offspring

In this thesis we demonstrated that intrauterine exposure to maternal apoE-deficiency results in acquisition of a cardiovascular disease risk that persists until adulthood (Chapter 2,3,4). In addition, our data showed that the increased cardiovascular disease risk already manifested itself after a single challenge of the vasculature by collar placement. We postulate that intrauterine environment poses a major risk factor for adult cardiovascular disease in humans.

The prospective follow-up study as suggested for pregnant women to identify a high-risk group of women should be extended by life-long follow-up of the progeny. In this way it can be determined whether intrauterine exposure to an adverse intrauterine environment actually results in an increased number of cardiovascular events in the offspring, such as myocardial infarction and ischemic stroke. Cardiovascular disease risk over time in the offspring can be assessed by regular examination of the levels of cholesterol and inflammation markers in the blood. In addition, noninvasive techniques for visualization of development and progression of atherosclerosis could be used. Ultrasound is frequently used to monitor intima-media thickness in the carotid artery. Transthoracic Doppler

echocardiography,⁸⁸ electron beam computerized tomography,⁸⁹ and MRI could be applied for assessment of coronary artery disease.

The society we live in nowadays is not beneficial for our health, and that of our children. Risk enhancers for cardiovascular disease lie in wait everywhere. Since fast food is much cheaper than vegetables, and eating microwave pizza much less time consuming than cooking, the food that we consume is not well-balanced. In addition, playing computer games and watching TV seems to be more attractive to children than playing outside or joining a soccer team. This may all sound a little bit far-fetched, but obesity in children age 2-19 years has increased over the last 20 years and only approximately 50% of children meet the Dutch standard for 'gezond bewegen' (CBS: Gezondheid en zorg 2007). Unfortunately, diet and physical activity are not the only risk factors. Since most of them are related to lifestyle, the primary goal for health care professionals should not be to keep cardiovascular disease risk low, but to get it low! Awareness on this issue needs to be achieved in parents, as well as children. In this way, the detrimental consequences of an adverse intrauterine environment on cardiovascular disease risk hopefully can be kept to a minimum.

The question arises to what extent children and adolescents that have been exposed to an adverse intrauterine environment can be treated. And where do we have to treat them for? In case children are diagnosed with hypercholesterolemia, mostly associated with heterozygous or homozygous familial hypercholesterolemia, statin treatment can be safely started.⁹⁰ However, maternal hypercholesterolemia does not result in hypercholesterolemia in children.⁹¹ Treatment with statins from an early age onwards for that purpose therefore seems not appropriate. When we consider the pleiotropic effects other than cholesterol-lowering, such as the anti-inflammatory effects, treatment could be beneficial. We have to realize that children exposed to an adverse intrauterine environment do not have symptoms for the condition for which they are treated. Therapy adherence therefore will be a serious problem.⁹² A recent study reported that as many as 15-60% of patients terminated statin treatment during the first 6 months after initial prescription.⁹³ Long-term compliance for treatment strategies that comprises daily use of medication most likely can not be achieved.

Conclusions

In this thesis we demonstrate that susceptibility for adult atherosclerosis can already be programmed during embryonic development. The indication that an adverse intrauterine environment, that comprises hypercholesterolemia, increased oxidative stress and inflammation, may have impact on the development and

progression of cardiovascular disease decades later hopefully triggers many researchers to add to, and to continue this work.

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Summary

Summary

In this thesis, the long-term consequences of an adverse intrauterine environment, created by maternal apoE-deficiency or Ldlr-deficiency, on adult atherosusceptibility in the offspring has been explored. In addition, the epigenetic mechanisms underlying the acquired susceptibility for cardiovascular disease were investigated.

Chapter 1 provides a general introduction on cardiovascular disease and the major environmental risk factors involved in atherosclerosis. Furthermore, the possible fetal origin of cardiovascular disease and the role of epigenetic programming in this process are described.

In **Chapter 2**, the effect of maternal apoE-deficiency, characterized by hypercholesterolemia, high oxidative stress and inflammation, on fetal vascular development and adult cardiovascular disease risk in apoE^{+/-} offspring has been investigated. In late fetal stages, a slight, but significant plasma cholesterol elevation was found in apoE^{+/-} fetuses from apoE^{-/-} mothers in comparison to apoE^{+/-} fetuses from wild-type mothers. In addition, morphological and morphometric analysis revealed profound vascular injury, characterized by a significant increase in loss of endothelial cell volume, in the carotid arteries of fetuses from apoE^{-/-} mothers. After birth, the damage to the endothelial cell lining was repaired and no pathology could be detected. Constrictive collar placement around the left common carotid artery of adult apoE^{+/-} offspring from apoE^{-/-} mothers which were fed a cholesterol-containing diet, resulted in severe neointima formation in 9 out of 10 mice analyzed. In apoE^{+/-} offspring from wild-type mothers, we only detected minor lesion volume (2 out of 10). Since neointima formation in apoE^{+/-} offspring from apoE^{-/-} mothers was accelerated and aggravated in relation to genetically identical offspring from wild-type mothers, it can be concluded that maternal apoE-deficiency during pregnancy has adverse effects on cardiovascular disease risk in the adult offspring.

Chapter 3 also concerned the apoE^{+/-} mouse model. Here we investigated whether adult apoE^{+/-} offspring from apoE^{-/-} mothers was also more susceptible to neointima formation in absence of a cholesterol-containing diet. On a chow diet, apoE^{+/-} offspring from apoE^{-/-} mothers, as well as apoE^{+/-} offspring from wild-type mothers were normocholesterolemic. The results showed that intrauterine exposure to maternal apoE-deficiency was sufficient to allow collar-induced neointima formation, even without cholesterol feeding. The neointimal lesions detected in

apoE^{+/-} offspring from apoE^{-/-} mothers were small, but present, sustaining the hypothesis that an adverse intrauterine environment provided by maternal apoE-deficiency poses a major risk factor for adult disease.

In **Chapter 4** the role of maternal hypercholesterolemia in intrauterine programming of adult cardiovascular disease risk was studied in the Ldlr^{-/-} mouse model. In Ldlr^{-/-} offspring, intrauterine exposure to maternal hypercholesterolemia resulted in fetal intimal thickening and profound spontaneous atherosclerosis in the adult stage. Maternal hypercholesterolemia appears to have limited effects on athero-susceptibility in Ldlr^{+/-} offspring. Analysis of the fetal vasculature and cholesterol levels revealed no adverse effects when compared with Ldlr^{+/-} fetuses from Ldlr^{-/-} mothers. In addition, collar placement in adult Ldlr^{+/-} offspring from Ldlr^{-/-} mothers did not accelerate and aggravate neointima formation in relation to Ldlr^{+/-} offspring from Ldlr^{+/-} mothers. Here, no lesions could be induced at all. Solely in spontaneous atherosclerosis in Ldlr^{+/-} mice from Ldlr^{-/-} mothers, a difference in aortic medial hypertrophy was detected suggesting that maternal hypercholesterolemia is not the primary trigger for intrauterine programming. In conclusion, we can state that most likely maternal hypercholesterolemia has a more regulatory role in this process.

In **Chapter 5** the capacity of adult endothelial cells to undergo endothelial-to-mesenchymal transformation in vivo was explored. Morphological analysis of femoral arteries of Tie2LacZ mice 3 days after nonconstrictive collar placement revealed endothelial detachment and proliferation. As early as 7 days after collar placement, β-galactosidase, which is endothelial cell-specific, could also be detected in the neointima. A number of these β-galactosidase-positive mesenchymal cells gained positivity for α-smooth muscle actin. The β-galactosidase-positive, α-smooth muscle actin-positive mesenchymal cells most likely are of endothelial cell origin. The contribution of these cells to the developing neointima suggests a role for endothelial cell derivatives in the development and progression of cardiovascular disease.

Chapter 6 is a review that focuses on the maternal signals that may cross the placental barrier and exert detrimental effects on embryonic and fetal development with a subsequent cardiovascular disease risk. Fetal undernutrition, maternal hypercholesterolemia and a number of other maternal factors all have been associated with an increased risk for cardiovascular events. Alterations in normal programming during sensitive embryonic or fetal developmental periods may result in the establishment of increased atherosclerosis susceptibility that persists into

adult life. Growing evidence from animal studies indicates that the underlying epigenetic mechanisms comprise DNA methylation or histone modifications.

Chapter 7 shows that profiles of histone triple-methylation modifications of lysines and the accompanying lysine methyltransferases in endothelial cells and smooth muscle cells were clearly differentially affected in apoE^{+/-} offspring from apoE^{-/-} mothers in relation to apoE^{+/-} offspring from wild-type mothers. Thus, it seems that intrauterine programming of susceptibility for cardiovascular disease in adult life is (in part) established by adverse influences of maternal apoE-deficiency on chromatin remodeling in the vasculature of the offspring.

Chapter 8 provides a general discussion of the data presented in this thesis. The role of maternal hypercholesterolemia, oxidative stress, and alterations in the immune system in prenatal programming of adult cardiovascular disease is discussed. In addition, the epigenetic mechanisms through which these risk factors may exert their effects are described. Finally, where possible, a link with the human situation is made and the implications of the data presented in this thesis on prevention and treatment strategies in pregnant women and offspring are presented.

In summary, in this thesis it becomes clear that the intrauterine environment created by the mother during pregnancy not only has beneficial effects on the developing embryo / fetus. Although it is too early to draw definite conclusions, the first results of this research line show that maternal apoE-deficiency, in contrast to maternal Ldlr-deficiency, adversely affects the offspring, not only in late fetal stages but also in adult life. Our data indicate that the inflammatory status of the mother and the lack of maternal apoE itself may attribute to the increased cardiovascular disease risk observed in the adult offspring. Hypercholesterolemia and oxidative stress possibly play a more regulatory role. In a first attempt to elucidate the underlying mechanism we show that maternal apoE-deficiency leads to changes in the histone triple-methylation modifications in the vascular wall of the offspring. This can be considered an important lead that needs to be investigated further. It does not mean, however, that we are close to complete elucidation of the underlying mechanism. A lot of research is needed to accomplish this and it is needed. Why? The fact that a hit so early in life exerts negative effects on cardiovascular disease risk in adulthood is worrisome. If we could succeed in elucidating the exact role of epigenetics in this process and are able to translate these data to the human situation, possibly we could reduce the incidence of cardiovascular disease.

Samenvatting

Samenvatting

In dit proefschrift zijn de consequenties van een nadelige intrauteriene omgeving, gecreëerd door maternale apoE-deficiëntie of Ldlr-deficiëntie tijdens zwangerschap, op de gevoeligheid voor aderverkalking in de volwassen nakomelingen bestudeerd. Tevens zijn de epigenetische mechanismen die ten grondslag liggen aan de verworven gevoeligheid voor hart- en vaatziekten onderzocht.

In **Hoofdstuk 1** wordt algemene informatie gegeven over hart- en vaatziekten en de belangrijkste risicofactoren voor aderverkalking. Daarnaast wordt de mogelijke foetale oorsprong van hart- en vaatziekten en de rol van epigenetische programmering in dit proces beschreven.

In **Hoofdstuk 2** zijn de effecten van maternale apoE-deficiëntie tijdens zwangerschap in muizen, dat gekenmerkt wordt door hoog cholesterol, hoge oxidatieve stress niveaus en toegenomen ontsteking, op foetale bloedvatontwikkeling en het risico op hart- en vaatziekten in volwassen apoE^{+/-} nakomelingen bestudeerd. Tijdens de laat foetale ontwikkeling werd een kleine, maar significante verhoging in het plasma cholesterol niveau gevonden in apoE^{+/-} foetussen van apoE^{-/-} moeders in vergelijking met apoE^{+/-} foetussen van wildtype moeders. Bovendien lieten morfologische en morfometrische analyses ernstige schade aan de vaatwand zien, gekarakteriseerd door een significante toename van verlies van endotheelcel volume in de halsslagaders van de foetussen van apoE^{-/-} moeders. Na de geboorte is de schade aan de endotheelcellaag hersteld en werd geen aderverkalking gevonden. De apoE^{+/-} nakomelingen kregen een dieet dat cholesterol bevatte om het cholesterolniveau in het bloed te verhogen. Op een leeftijd van 16 weken werd een kokertje rondom de linker halsslagader van volwassen apoE^{+/-} nakomelingen geplaatst. Het kokertje vernauwt het bloedvat gedeeltelijk waardoor neointima vorming wordt opgewekt. Cuff-plaatsing in apoE^{+/-} nakomelingen van apoE^{-/-} moeders resulteerde in ernstige neointimavorming in 9 van de 10 nakomelingen. In apoE^{+/-} nakomelingen van wildtype moeders werden kleine verdikkingen gevonden in 2 van de 10 nakomelingen. Neointimavorming in apoE^{+/-} nakomelingen van apoE^{-/-} was versneld en verergerd in verhouding tot genetisch identieke apoE^{+/-} nakomelingen van wildtype moeders. We concluderen dat maternale apoE-deficiëntie tijdens zwangerschap nadelige effecten heeft op het risico voor hart- en vaatziekten in de volwassen nakomelingen.

In **Hoofdstuk 3** werd ook het apoE^{+/-} muismodel gebruikt. We hebben onderzocht of volwassen apoE^{+/-} nakomelingen van apoE^{-/-} moeders ook gevoelig zijn voor neointimavorming in afwezigheid van een cholesteroldieet. Een normaal dieet zonder toevoeging van cholesterol resulteerde in normale cholesterolwaarden in apoE^{+/-} nakomelingen van zowel apoE^{-/-} moeders als wildtype moeders. De resultaten toonden aan dat blootstelling aan maternale apoE-deficiëntie tijdens de embryonale ontwikkeling voldoende was om neointimavorming op te wekken via cuff-plaatsing, zelfs zonder een cholesteroldieet. De neointima verdikkingen in apoE^{+/-} nakomelingen van apoE^{-/-} moeders waren klein, maar aanwezig. Dit bevestigt de hypothese dat een nadelige intrauteriene omgeving gecreëerd door maternale apoE-deficiëntie een belangrijke risicofactor is voor ziekte in het volwassen leven.

Hoofdstuk 4 beschrijft de rol van maternaal hoog cholesterol in intrauteriene programmering van het risico op hart- en vaatziekten in volwassen nakomelingen. Hiervoor werd het Ldlr^{-/-} muismodel gebruikt. In Ldlr^{-/-} nakomelingen leidde intrauteriene blootstelling aan maternaal hoog cholesterol tot vorming van intimaverdikkingen in de bloedvaten van de foetus en ontwikkeling van aderverkalking in het volwassen leven. Maternaal hoog cholesterol lijkt weinig effect te hebben op de gevoeligheid voor aderverkalking in Ldlr^{+/-} nakomelingen. Bestudering van de foetale bloedvaten en cholesterolniveaus in Ldlr^{+/-} foetussen van Ldlr^{-/-} moeders lieten geen nadelige effecten zien in vergelijking met Ldlr^{+/-} foetussen van Ldlr^{+/-} moeders. Bovendien leidde cuff-plaatsing in volwassen Ldlr^{+/-} nakomelingen van Ldlr^{-/-} moeders niet tot versnelde en verergerde neointimavorming. Neointimavorming kon zelfs niet opgewekt worden. Alleen in spontane ontwikkeling van aderverkalking werd een toename in hypertrofie van de mediale laag van de aorta gevonden in Ldlr^{+/-} nakomelingen van Ldlr^{-/-} moeders. Deze data suggereren dat maternaal hoog cholesterol niet de primaire veroorzaker is van intrauteriene programmering. We kunnen concluderen dat maternaal hoog cholesterol waarschijnlijk een regulatoire rol heeft in dit proces.

In **Hoofdstuk 5** is het vermogen van volwassen endotheelcellen om te transformeren van endotheelcel naar mesenchymcel onderzocht. In Tie2LacZ muizen, waarbij door genetische manipulatie de endotheelcellaag met een blauwe kleur gemarkeerd is, werden niet-vernauwende kokertjes rondom de dijbeenslagaders geplaatst. Drie dagen na cuff-plaatsing werd loslating van endotheelcellen zowel als proliferatie van endotheelcellen gevonden. Vanaf 7 dagen na cuff-plaatsing kon de blauwe kleur behalve in de endotheelcellaag ook in de neointima gevonden worden. Een aantal blauwe mesenchymcellen werden ook

positief voor de marker α -gladde spiercel actine. De dubbel-positieve mesenchymcellen zijn waarschijnlijk van endotheelcel oorsprong. De bijdrage van deze cellen aan neointimavorming suggereert een rol voor endotheelcel afgeleide cellen in de ontwikkeling en progressie van hart- en vaatziekten.

Hoofdstuk 6 is een review over factoren van de moeder die via de placenta het kind kunnen bereiken en daar nadelige effecten op embryonale en foetale ontwikkeling kunnen uitoefenen. Dit kan vervolgens leiden tot een verhoogd risico op hart- en vaatziekten. Foetale ondervoeding, maternaal hoog cholesterol en een aantal andere factoren tijdens zwangerschap zijn geassocieerd met een verhoogde kans op manifestatie van hart- en vaatziekten in de nakomelingen, bijvoorbeeld hart- en herseninfarcten. Veranderingen in de normale programmering tijdens kritieke perioden van de embryonale of foetale ontwikkeling kan leiden tot de aanleg van een verhoogde gevoeligheid voor aderverkalking die blijft bestaan tot het volwassen leven. Toenemend bewijs uit dierstudies doet vermoeden dat DNA methylatie en modificatie van histonen epigenetische mechanismen zijn die ten grondslag liggen aan de aanleg van de gevoeligheid.

Hoofdstuk 7 laat zien dat de profielen van histon triple-methylatie modificaties van lysines en de bijbehorende lysine methyltransferases in endotheelcellen en gladde spiercellen veranderd zijn in apoE^{+/-} nakomelingen van apoE^{-/-} moeders in vergelijking met apoE^{+/-} nakomelingen van wildtype moeders. Het lijkt waarschijnlijk dat intrauteriene programmering van een gevoeligheid voor hart- en vaatziekten in het volwassen leven (deels) wordt aangelegd door nadelige invloeden van maternale apoE-deficiëntie op de remodellering van chromatine in de bloedvaten van de nakomelingen.

Hoofdstuk 8 geeft een algemene discussie over de data gepresenteerd in hoofdstuk 2-7. De rol van maternaal hoog cholesterol, oxidatieve stress en veranderingen in het immuunsysteem in intrauteriene programmering van hart- en vaatziekten wordt besproken. Daarnaast worden de epigenetische mechanismen via welke de risicofactoren hun effecten uitoefenen beschreven. Tenslotte wordt waar mogelijk een link gelegd met de situatie in de mens. De mogelijke implicaties van de data beschreven in dit proefschrift op preventie- en behandelingsstrategieën in zwangere vrouwen worden besproken.

In samenvatting, in dit proefschrift wordt het duidelijk dat de intrauteriene omgeving gecreëerd door de moeder gedurende de zwangerschap niet alleen maar positieve effecten heeft op het ontwikkelende embryo. Ondanks dat het nog te vroeg is om

definitieve conclusies te trekken laten de eerste resultaten van het onderzoek zien dat maternale apoE-deficiëntie, in tegenstelling tot maternale Ldlr-deficiëntie, een nadelige invloed heeft op de nakomelingen, zowel in laat foetale stadia alsmede in volwassen leven. Onze resultaten suggereren dat de ontstekingsstatus en het gebrek aan apoE bij de moeder bijdragen aan het verhoogde risico op hart- en vaatziekten bij de volwassen nakomelingen. Hoog cholesterol en oxidatieve stress lijken een meer regulerende rol te spelen. In een eerste poging om het onderliggende mechanisme te ontrafelen laten we zien dat maternale apoE-deficiëntie leidt tot veranderingen in de histon triple-methylatie modificaties in de vaatwand van de nakomeling. Epigenetische mechanismen spelen dus waarschijnlijk een belangrijke rol in het vastleggen van de gevoeligheid voor hart- en vaatziekten. Het betekent niet dat we al dichtbij de complete ontrafeling van het onderliggende mechanisme zijn. Veel onderzoek is nodig om dit doel te bereiken en het is hard nodig. Waarom? Het feit dat blootstelling zo vroeg in het leven tientallen jaren later negatieve effecten heeft op het risico voor hart- en vaatziekten is zorgwekkend. Indien we succesvol zijn in het ontrafelen van de exacte rol van epigenetica in dit proces en in staat zijn de gegevens te vertalen naar de humane situatie, is het wellicht mogelijk de incidentie van hart- en vaatziekten te verminderen.

Curriculum Vitae

Fanneke Elizabeth Alkemade was born in Leiden, The Netherlands, at February 19th 1981. She graduated from the Northgo College in Noordwijk in 1999 (Atheneum). After that, she started her study Biomedical Sciences at the Leiden University Medical Center. She received her Bachelor's degree in december 2002. During her Master education she performed two research internships. The first was carried out at the Department of Neurology of the Leiden University Medical Center, under supervision of Dr. Jan J.G.M. Verschuuren, and was entitled 'Lambert-Eaton myasthenic syndrome (LEMS): Differences in antibody binding to the voltage gated calcium channel (VGCC) by tumour versus non-tumour patients'. Her graduation research project was performed at the Department of Anatomy and Embryology of the Leiden University Medical Center under supervision of Prof. Dr. Robert E. Poelmann and Dr. Beerend P. Hierck. This training period was entitled 'Shear stress in the chicken embryo endothelium'. In January 2004 she received the 'Heart of Biomedical Science' Award for the best research proposal within the Master education biomedical sciences. She received her Master's degree *cum laude* in August 2004.

Also in August 2004, she started her PhD-project at the Department of Anatomy and Embryology of the Leiden University Medical Center on the fetal origin of adult atherosclerosis. During this PhD-project she performed experiments in collaboration with the Departments of Human Genetics, Nephrology, Endocrinology, Immunohematology and Blood Transfusion of the Leiden University Medical Center and TNO Quality of Life. The results of the study are presented in this thesis.

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List of Publications

Alkemade FE, Willems van Dijk K, VanMunsteren JC, Hierck BP, Havekes LM, Gittenberger-de Groot AC, DeRuiter MC. Maternal apoE-deficiency promotes collar-induced neointima formation in adult apoE⁺/⁻ offspring even without cholesterol feeding. *Submitted for publication*.

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