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

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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^{o}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 ± 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 normocholesteromia (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, hypercolesterolemia; 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 ($^{\dagger}P < 0.000$).

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 mm^3) 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 mm^3). All estimated volumes were comparable to $Ldlr^{+/+}$ controls (0.059 ± 0.016 mm^3).

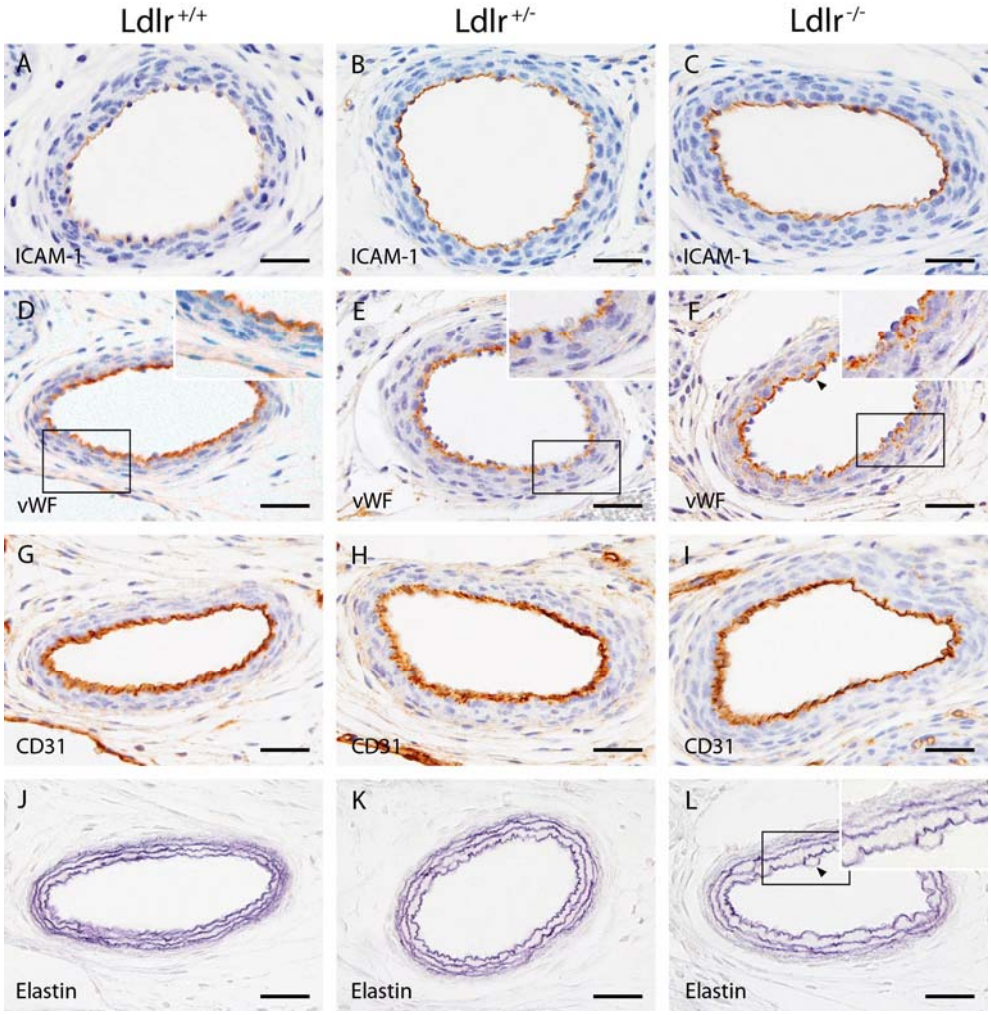

Figure 1. Morphology of fetal carotid arteries. Cross-sections from (A,D,G,J) Ldlr$^{+/+}$, (B,E,H,K) Ldlr$^{+/-}$ from Ldlr$^{+/-}$ mothers and (C,F,I,L) Ldlr$^{-/-}$ fetuses from Ldlr$^{-/-}$ mothers at E17.5. Because identical staining patterns were found in Ldlr$^{+/-}$ from Ldlr$^{-/-}$ mothers 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 µm.

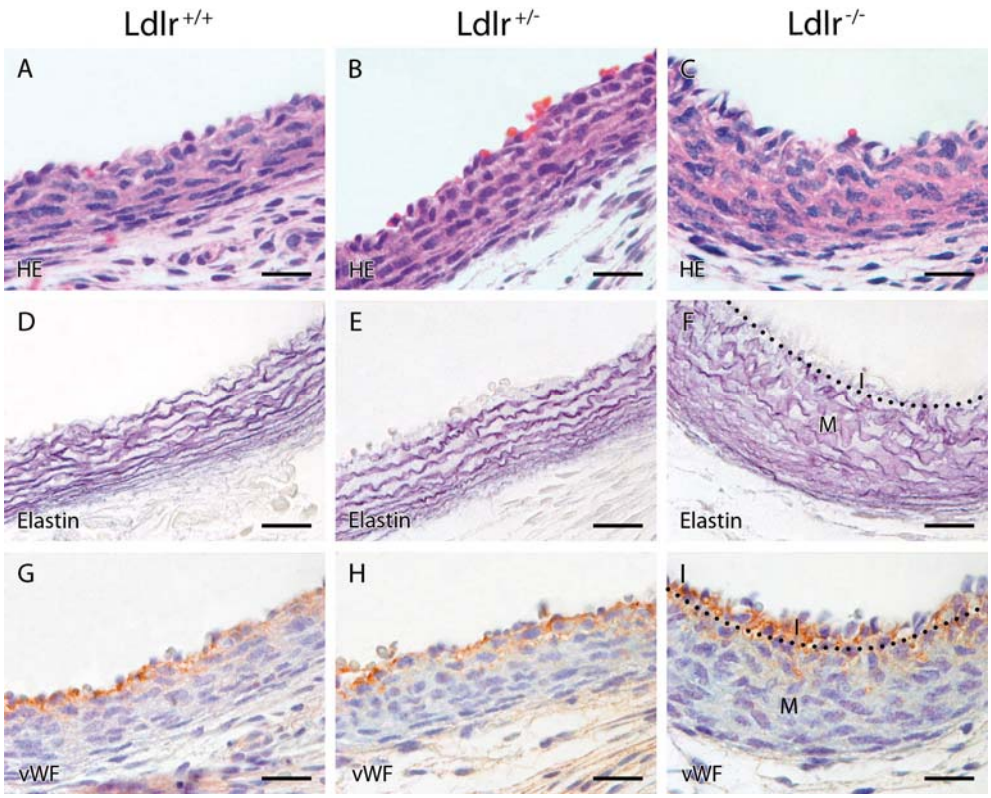

Figure 2. Morphology of fetal ascending aorta. Cross-sections from (A,D,G) Ldlr$^{+/+}$, (B,E,H) Ldlr$^{+/-}$ from Ldlr$^{-/-}$ mothers and (C,F,I) Ldlr$^{-/-}$ fetuses from Ldlr$^{-/-}$ mothers with (A-C) general HE staining, (D-F) elastin and (G-I) anti-vWF. Section B,E,H also represent Ldlr$^{+/-}$ 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 µm.

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 mm^3).

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 mm^3, $P = 0.016$, Figure 3C). Vascular lumen volume was maintained (1.66 ± 0.14 versus 1.69 ± 0.08 mm^3).

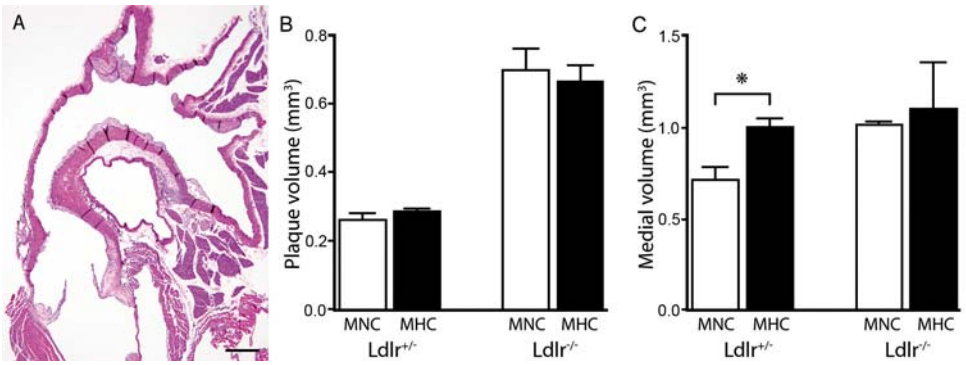

Figure 3. Effects of in utero exposure of $Ldlr^{+/-}$ and $Ldlr^{-/-}$ mice on spontaneous atherosclerosis in adult life. (A) Representive cross-section of the adult aorta showing the sites of atherogenesis; the aortic root, the inner curvature of the aortic arch and the areas upstream of the branch points. Scale bar: 300 µm. 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 ± 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 colleagues[20] 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 colleagues[23]). 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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