Maternal Hypercholesterolemia and Treatment During Pregnancy Influence the Long-Term Progression of Atherosclerosis in Offspring of Rabbits

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Abstract—Maternal hypercholesterolemia during pregnancy is associated with enhanced fatty streak formation in human fetuses and faster progression of atherosclerosis during childhood even under normocholesterolemic conditions. A causal role of maternal hypercholesterolemia in lesion formation during fetal development has previously been established in rabbits. The same experimental model is now used to establish that maternal hypercholesterolemia or ensuing pathogenic events in fetal arteries enhance atherogenesis later in life. Five groups of rabbit mothers were fed chow, cholesterol-enriched chow, or cholesterol-enriched chow plus 1000 IU vitamin E, 3% cholestyramine, or both during pregnancy. Offspring of all groups (n = 136) were fed a mildly hypercholesterolemic diet for up to a year and had similar cholesterol levels. Aortic lesion sizes and lipid peroxidation products in plasma and lesions in offspring were determined at birth, 6 months, or 12 months. Lesion progression in offspring of hypercholesterolemic mothers was greater than in all other groups. At each time point, offspring of hypercholesterolemic mothers had 1.5- to 3-fold larger lesions than offspring of normocholesterolemic mothers (P<0.01), with the greatest absolute differences at 12 months. Maternal treatment reduced lesions by 19% to 53%, compared with offspring of untreated hypercholesterolemic mothers (P<0.01), with the greatest effect in the vitamin E groups. At 12 months; lesions in offspring of all vitamin E and cholestyramine-treated mothers were similar to those of normocholesterolemic mothers. Lipid peroxidation end-products in lesions and plasma showed analogous differences between groups as lesions (P<0.01). Thus, pathogenic programming in utero increases the susceptibility to atherogenic risk factors later in life and maternal intervention with cholesterol-lowering drugs or antioxidants reduce postnatal lipid peroxidation and atherosclerosis in their offspring. (Circ Res. 2001;89:●●●●●●●.)

Key Words: pathogenesis ■ fetus ■ prevention ■ oxidation ■ vitamin E ■ cholestyramine

Increasing evidence suggests that maternal hypercholesterolemia during pregnancy triggers pathogenic events in the fetal aorta and that these may influence atherogenesis later in life. Formation of fatty streaks, the earliest stages of atherosclerotic lesions, already begins during fetal development.1,2 The distribution of fetal lesions in the aorta reflects that of adult atherosclerosis. Maternal hypercholesterolemia—even when temporary and limited to pregnancy—is associated with greatly enhanced fatty streak formation.1,2 During the second trimester, fetal plasma cholesterol levels are high and proportional to the maternal cholesterol levels.3 However, they decline with increasing fetal age1 and are very low at term birth. Despite this, fetal lesions regress only partially toward the end of pregnancy or during infancy, when cholesterol levels are normal.3 The Fate of Early Lesions in Children (FELIC) study, which determined the extent of atherosclerosis in the aorta of children (1 to 14 years old) at autopsy, demonstrated that atherosclerosis progresses much faster in offspring of hypercholesterolemic mothers than in offspring of normocholesterolemic mothers,3 even under conditions of normocholesterolemia. This difference in atherogenesis could not be accounted for by conventional risk factors of the disease. We, therefore, hypothesized that pathogenic events occurring in the fetal artery (as a result of maternal or fetal hypercholesterolemia) enhance the susceptibility to atherosclerosis later in life.3,4 We also postulated that lipid oxidation plays an important pathogenic role in fetal lesion formation and its postnatal consequences, because oxidation of LDL is prevalent in fetal lesions and because a number of pathways regulating the expression of genes modulating atherogenesis are oxidation-sensitive.5–12 An important corollary of this hypothesis is that lipid-lowering and antioxidant interventions in mothers during pregnancy should provide long-lasting benefits to their offspring.
Because of the genetic heterogeneity of human population and the likelihood that genetic differences between normo- and hypercholesterolemic mothers contribute to enhanced atherogenesis in humans, genetically relatively homogeneous animal models are required to establish the causality of maternal hypercholesterolemia in fetal lesion formation and to determine its influence on the susceptibility to atherosclerosis later in life. A recent study in New Zealand White (NZW) rabbits showed that diet-induced maternal hypercholesterolemia during pregnancy is sufficient to markedly enhance fetal lesion formation. It also provided direct evidence for a causal role of hyperlipidemia and lipid oxidation in the fetal onset of atherogenesis by demonstrating that lipid-lowering (cholestyramine) or antioxidant (vitamin E) treatment of mothers during pregnancy significantly reduces the size of lesions in their offspring at birth. However, to date it has not been experimentally established that maternal hypercholesterolemia significantly affects the extent of postnatal atherogenesis, that maternal treatment reduces lesion formation later in life, and that these effects remain relevant in the presence of conventional risk factors of atherosclerosis in offspring, such as hypercholesterolemia. We now provide this evidence in the NZW model.

Materials and Methods

Rabbits

Five groups of 4- to 5-month-old female NZW rabbits (Nossan) were fed either regular chow (4% fat, 18% protein, 60% carbohydrate, Control group); a cholesterol-enriched diet (Teklad adjusted calories diet; 9% fat, 19% protein, 55% carbohydrate, 4% fibers, to which 0.25% cholesterol dissolved in ether was added; previously termed Chol2 in Napoli et al), which results in total plasma cholesterol (TC) levels of about 350 mg/dL (Hyperchol group); or the same hypercholesterolemic diet supplemented with 1000 IU of vitamin E (α-tocopherol; Sigma) (Hyperchol+VitE group), 3% cholestyramine (Hyperchol+Cholestyr group), or both vitamin E and cholestyramine (Hyperchol+VitE+Cholestyr group). A much higher dose of vitamin E than in the preceding study (100 IU) was used to determine the maximum degree of protection achievable. Because our preceding study in this model had established that fetal lesions do not progress at all (at least during the first 6 months) in any group of offspring fed regular chow, no additional control groups were included in the present experiment (eg, offspring of normo- or hypercholesterolemic mothers fed regular chow after birth). Diets were started 2 weeks before mating and continued until 1 week postpartum. Male breeders were untreated. TC levels were determined at week 2 and 3 of pregnancy by a standard enzymatic method. Part of each litter were sacrificed at birth. The remaining males were weighed at 4 weeks and fed a standard diet (Teklad) containing 0.14% cholesterol for about 6 months or 12 months. Because our previous study had indicated that combination treatment with both vitamin E and cholestyramine only marginally increased the protective effect at birth compared with single treatment, offspring of this group were only studied at 12 months. Experimental groups contained roughly equal numbers of males and females and data for both sexes were analyzed together because previous studies had not indicated significant gender differences. All experiments were performed under approved institutional animal protocols.

Tissue Preparation, Quantification of Atherosclerosis, and Immunohistochemistry

Aortas were perfused and cryosections prepared as previously described. Lesion sizes were determined by computer-assisted image analysis of 25 to 30 equidistant frozen Oil red O-stained sections each from the aortic arch, thoracic, and abdominal aorta. Additional aortic segments (0.8 to 1 mm long) were pooled as lesion or nonlesion tissue. Results are reported as the average cumulative lesion area per section. All determinations of atherosclerosis were performed by the same investigator blinded to the identity of the groups. In addition, lesions at 12 months were determined as percentage of the aortic surface area covered by Oil red O-positive atherosclerotic lesions before sectioning them. Immunocytochemistry with monoclonal antibodies to oxidation-specific epitopes, apolipoprotein B, and macrophages was performed as previously described. Peroxidative End-Products

Fatty acids (FAs) were isolated from plasma or aortic homogenates, as described, and concentrations of 10-hydroxy oleic acid, 12-hydroxy linoic acid, and 10-hydroxy arachidonic acid determined by a combination of gas chromatography and mass spectrometry, as previously described. The concentration of oxidized FAs in atherosclerotic lesions of each rabbit was determined by comparison of pooled normal and lesion tissue, as previously described. Plasma malondialdehyde (MDA) content was measured as thiobarbituric acid reactive substances. In maternal plasma, these parameters were measured at the 2nd and 3rd week of pregnancy; in their offspring, plasma and aortic measurements were performed at euthanasia.

Statistical Analysis

Results are presented as mean±SEM. Comparisons between groups determined by unpaired Student’s t test. Probability values reported were Bonferroni-corrected, unless otherwise indicated.

Results

Body weights of mothers and offspring were not significantly affected by diets. Similarly, there was no difference in malformations in offspring between groups. Plasma cholesterol levels of mothers and their offspring are shown in Figure 1. Aas expected, the hypercholesterolemic diet markedly raised maternal TC levels from 65 mg/dL in the Control group to about 330 mg/dL in the Hyperchol group. Cholestyramine treatment significantly lowered maternal TC to 190 mg/dL. Vitamin E had no significant effect on TC. Offspring of all groups had similar, low TC levels at birth (46 to 48 mg/dL). The mildly hypercholesterolemic diet fed after weaning raised TC in all four groups to 149 to 153 mg/dL at 6 months and 262 to 284 mg/dL at 12 months. These levels were significantly higher than those at birth (P<0.0001), but again there were no significant differences between groups at either time. Data of the Vit E+Cholestyr group are not included in Figure 1 because of the small numbers of mothers (n=2) and the fact that offspring were only studied at 12 months. The two mothers of this group had similar TC values as the Chol+Cholestyr group, and their offspring at 12 months had similar TC (284±11 mg/dL) as all other groups.

Maternal plasma levels of MDA (Figure 2, left panel), 12-OH linoic acid (Figure 2, right panel), and 10-OH oleic acid (not shown) during pregnancy were significantly elevated in hypercholesterolemic rabbits, compared with controls. Treatment with 1000 IU vitamin E markedly reduced all three plasma lipid peroxidation parameters, often to below the levels of the control group, whereas cholestyramine only resulted in a minor decrease of MDA. Maternal plasma levels of lipid peroxidation products, in particular MDA, correlated well with the extent of lesions in offspring at birth (r=0.85,
Lesion sizes in offspring of the four main groups are shown in Figure 3. Results at birth were comparable to those previously obtained. Lesions at birth were significantly greater in the Hyperchol group than in the Control group. Maternal treatment with either vitamin E or cholestyramine significantly reduced lesion sizes. The size of lesions in the Hyperchol+Vit E group was also significantly smaller than in the Hyperchol+Cholestyr group, although maternal plasma cholesterol levels were much higher in the former. Differences between groups at 6 months were also significant, but unexpectedly, lesion sizes were only moderately larger than those of the respective groups at birth (P<0.0001). In contrast, there was a 5.5- to 8.3-fold increase in atherosclerosis during the following six months (P<0.0001 in all groups). Lesions in the Vit E or Cholestyr groups were not significantly different from the control group. Lesions in the Vit E+Cholestyr group (617±41×10^2 μm/section, not shown in Figure 3) were slightly, but not significantly, smaller than those of the Control and the other two treatment groups. The absolute differences between the Hyperchol and all other groups were much greater at 12 months than at earlier time points. When data at 12 months were compared with those at 6 months (Inset of Figure 3) or birth (not shown), progression of atherosclerosis (indicated by the slope of the lines) was much faster in offspring of the Hyperchol group than in all other groups. Immunohistochemistry revealed differences in the lesion content of native and oxidized LDL and macrophages between groups consistent with the differences in lesion size (data not shown).

In addition to measuring lesions in equidistant cross-sections through the aorta (the same parameter determined in human fetuses and children), atherosclerosis at 12 months was also determined in Oil red O-stained en face preparations of the aortas (before sectioning), in order to facilitate comparison with other studies expressing lesions as percent of atherosclerotic surface area (Figure 4, upper panel). Results were qualitatively similar to those obtained for cross-sectional lesion areas and significant in standard t tests, but only the Vit E and Vit E+Cholestyr groups remained

Figure 1. Average plasma cholesterol of mothers during pregnancy and terminal plasma cholesterol of offspring euthanized at birth or after 5 or 11 months on a mildly hypercholesterolemic diet (ie, at age 6 and 12 months). Hyperchol indicates hypercholesterolemic diet. Note that all diets and treatments in the group names refer to mothers, not their offspring. *P<0.0001 vs Control; **P<0.0001 vs Hyperchol; ***P<0.0001 vs Hyperchol and Vit E. Data are mean±SEM of 5 mothers/group, 11 to 16 rabbits/group at birth and 6 months, and 4 to 6 rabbits/group at 12 months. The smaller group sizes at 12 months were due to the expectation that differences in atherogenesis would be greatest at the later time point. All probability values are Bonferroni-corrected. Differences between offspring of different ages were significant at P<0.0001 for all groups (not indicated in figure).

Figure 2. Maternal plasma levels of malondialdehyde (MDA) and oxidized (10-OH) linoleic acid. Hyperchol indicates hypercholesterolemic diet. All diets and treatments in the group names refer to mothers, not their offspring. ***P<0.01 vs Control; ****P<0.05 vs Control; #P<0.0001 vs Hyperchol; $P<0.0001$ vs Hyperchol+Cholestyr; n=5 in all groups.
significant ($P<0.05$) after Bonferroni correction. Examples of stained aortas are provided in the lower panel of Figure 4.

Measurement of several parameters of lipid peroxidation in the aortic intima revealed significant differences between groups at each time point and an absolute increase over time in each group, consistent with the increasing lesion size. Data for one of the parameters, 12-OH linoleic acid, are shown in Figure 5. Similar results were obtained for the intimal concentration of oxidized (10-hydroxy) oleic acid and MDA (not shown). The difference between the Hyperchol group and the Control and Vit E groups, as well as the Vit E+Cholestyr group (6.05±0.19×10^2 ng/mg; not shown in Figure 5), also increased in absolute terms over time. This is indicative of reduced LDL oxidation in offspring of antioxidant-treated mothers.

Significant differences between groups were seen in the plasma levels of MDA (Figure 6). Although such differences are not surprising at birth because of the potential placental permeability for maternal oxidized fatty acids, their observation at 6 and 12 months was unexpected, given the absence of dietary differences and virtually identical plasma cholesterol levels. Differences in plasma levels of lipid peroxidation products between the offspring of hypercholesterolemic and vitamin E–treated mothers were similar at 6 and 12 months, despite the substantial increase in atherosclerosis during this period. This may indicate persistent effects of maternal treatment on postnatal lipid peroxidation.

Because of the controversy regarding the dose of vitamin E required to achieve protection in humans and in view of recent recommendations of 400 IU vitamin E/d from the American Heart Association/American College of Cardiology and the European Society of Cardiology,15,16 a preliminary experiment was also performed with 2 mothers treated with 400 IU vitamin E and 4 offspring at 12 months. Compared with the Hyperchol group, this showed a marked reduction of lesions, both in terms of cross-sectional area ($876±28×10^3$ mm^2) and atherosclerotic surface area ($17.1±0.79\%$) ($P<0.01$ versus Hyperchol for both), and a significant reduction of oxidized linoleic acid in lesions ($7.32±0.37×10^{-2}$ ng/mg; $P<0.001$), but only a marginal reduction of plasma MDA (0.31±0.012 mmol/L; NS). Thus, 400 IU vitamin E conveyed significant protection but consistently less than 1000 IU.

**Discussion**

The present study demonstrates that pathogenic events in fetal arteries associated with maternal hypercholesterolemia are capable of enhancing the susceptibility to atherosclerosis later in life, and that cholesterol-lowering interventions or antioxidant treatment of hypercholesterolemic mothers during pregnancy have long-term beneficial effects in their offspring.

As previously demonstrated in the same rabbit model,13 diet-induced maternal hypercholesterolemia during pregnancy markedly increased fatty streak formation during fetal development, whereas maternal treatment with vitamin E or cholestyramine significantly reduced lesion sizes in their offspring, compared with untreated mothers. In the present study, a higher dose of vitamin E (1000 IU) was used than in the preceding one (100 IU). This yielded a greater reduction of lesion sizes and intimal content in lipid peroxidation products at birth. After weaning and five months on the mildly hypercholesterolemic diet, lesion sizes in aortic cross-sections showed only a moderate increase. At 12 months, hypercholesterolemia was more extensive and lesions increased up to 8.3-fold, compared with 6 months. Lesion...
progression was faster in offspring of untreated hypercholesterolemic mothers than in offspring of normocholesterolemic mothers. The absolute difference in atherosclerosis between offspring of hypercholesterolemic mothers and that of all treatment groups was also greatest at 12 months. Vitamin E (alone or in combination with cholestyramine) was most protective, but lesions in all treatment groups were reduced to a level similar to that of the control group, whose mothers did not receive the hypercholesterolemic diet. Measurements of the atherosclerotic surface area at 12 months confirmed the protective effect of antioxidant and lipid-lowering interventions. Together with previous data showing no progression of lesions in offspring of chow-fed NZW rabbits up to at least 6 months of age, these results establish that maternal hyper-
cholesterolemia enhances the atherogenic response to postnatal hypercholesterolemia. However, it remains to be determined whether the acceleration of lesion formation increases with increasing postnatal plasma cholesterol or whether very high cholesterol levels conversely mask some of this effect.

The preceding study demonstrated (and the present data at birth confirm) that maternal hypercholesterolemia enhances lesion formation during the fetal development. The mechanisms mediating the atherogenic stimulus from mothers to fetuses remain to be elucidated, but increasing data suggest that maternal hypercholesterolemia may increase fetal cholesterol levels during part of the gestation period. For example, in humans, maternal and fetal plasma cholesterol levels correlate up to about the 6th month of fetal development. Recent evidence in hamsters also supports the notion that maternal sterol metabolism influences the fetal one. The protective effect of vitamin E treatment established that enhanced lipid oxidation also contributes to fetal lesion formation, although it is not clear whether the reduction of fetal lesions is due to a direct effect of vitamin E in the fetal artery, or whether it stems from increased antioxidant protection in the mother. Thus, it is tempting to assume that hypercholesterolemia and enhanced LDL oxidation promote fetal fatty streak formation by the same mechanisms by which they enhance conventional atherogenesis. The correlation between the plasma concentration of lipid peroxidation products and lesions at birth also suggests that the former may be a good indicator of lesion formation during fetal development.

Given the small size of fetal arteries and lesions and the fact that fetal fatty streaks may partially regress, fetal lesion formation and the factors promoting it would be of little clinical relevance unless they significantly enhance atherogenesis later in life. The present study establishes that maternal hypercholesterolemia is in fact associated with accelerated postnatal atherogenesis, and that this can be greatly reduced or even prevented by interventions in mothers during pregnancy. Despite the apparent association between maternal hypercholesterolemia and accelerated postnatal atherogenesis, it is possible that the causal link is an indirect one, and that accelerated lesion formation later in life is the consequence of increased fetal lesion formation. We previously postulated that increased oxidative stress during fetal development would affect arterial gene expression and/or transcription and that some of these changes would persist. This was based on the observation of markedly increased lipid peroxidation in plasma and lesions of human mothers, fetuses, and newborns (now confirmed in the rabbit model), and on the fact that multiple nuclear signaling pathways are oxidation-sensitive. Microarray-based determinations in a murine model of fetal atherogenesis indicate that differences in arterial gene expression later in life indeed exist (C. Napoli, F. de Nigris, J. Welch, A. Li, F. Calara, Stuart, C.K. Glass, and W. Palinski, unpublished results). However, atherogene-

Figure 5. Oxidized linoleic acid in the aortic intima of offspring. Legends indicate maternal diet and treatment. Hyperchol indicates hypercholesterolemic diet. *P<0.0001, **P<0.01 vs Control, respectively; *P<0.0001, ***P<0.01 vs Hyperchol, respectively; P<0.0001 vs Hyperchol+Cholestyr (Bonferroni-corrected probability value).

Figure 6. Plasma levels of malondialdehyde (MDA) in offspring at euthanasia. Legends indicate maternal diet and treatment. Hyperchol indicates hypercholesterolemic diet; Cholestyr, cholestyramine. *P<0.0001, **P<0.001 vs Control, respectively; *P<0.0001, ***P<0.01 vs Hyperchol, respectively; **P<0.001, ***P<0.01 vs Hyperchol+Cholestyr, respectively (Bonferroni-corrected probability value).
sis may involve an extraordinary number of factors,23 including products of yet undefined genes as well as complex regulatory interactions between multiple factors, and the mechanisms responsible for increased susceptibility to postnatal atherosclerosis remain to be established.

Considering these results, it appears likely that maternal hypercholesterolemia or the ensuing increased fetal lesion formation also contribute to accelerated postnatal atherogenesis in humans. Previous studies by other groups amply demonstrated the occurrence and progression over time of atherosclerotic lesions in children and young adults,22–25 but did not assess the impact of maternal hypercholesterolemia. The FELIC study showed that atherosclerosis progresses much faster in offspring of human hypercholesterolemic mothers than in offspring of normocholesterolemic mothers throughout childhood and adolescence, even under conditions of normocholesterolemia.3 However, it could not provide experimental evidence for the causal role of maternal hypercholesterolemia in this acceleration because genetic differences are likely to exist between normocholesterolemic mothers and mothers with temporary or chronic hypercholesterolemia that may also predispose to increased atherosclerosis. Even now that such evidence has been obtained in a genetically relatively homogeneous rabbit model, it will be difficult to establish in humans to what extent maternal hypercholesterolemia per se is responsible for accelerated atherogenesis, in particular in the presence of conventional risk factors of the disease. In analogy, the degree of protection achievable by maternal treatment in humans is difficult to predict. In the present experiment, maternal treatment with 1000 IU of vitamin E alone or in combination with cholestyramine effectively abolished the atherogenic consequences of maternal hypercholesterolemia. Cholesterol levels of 270 mg/dL (12 months) are 5-fold higher than physiological levels in NZW rabbits, but much lower than those inducible by the standard 1% cholesterol diets used in many atherosclerosis studies in this model or the upper range of human cholesterol levels. It therefore remains to be seen whether extreme cholesterol levels, or combinations with other risk factors, do not overcome the protective effect. Similarly, it cannot be ruled out that more powerful atherogenic stimulation reduces the relative impact of maternal hypercholesterolemia and fetal lesion formation on later atherosclerosis.

Nevertheless, the present evidence linking fetal programming with accelerated atherogenesis later in life supports the notion that maternal hypercholesterolemia during pregnancy should be included among the risk factors predicting the disease and determining the need for more intense monitoring26 and treatment.27 Results also suggest that interventions in mothers may offer long-term benefits to their offspring, in particular treatment with vitamin E,28 which is considered safe during pregnancy, or newly developed lipid-lowering drugs.

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References


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