Maternal Hypercholesterolemia Enhances Atherogenesis in Normocholesterolemic Rabbits, Which Is Inhibited by Antioxidant or Lipid-Lowering Intervention During Pregnancy

An Experimental Model of Atherogenic Mechanisms in Human Fetuses

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Abstract—Maternal hypercholesterolemia during pregnancy is associated with a marked increase in aortic fatty streak formation in human fetuses and faster progression of atherosclerosis during normocholesterolemic childhood. However, the mechanisms responsible are unknown, and the contribution of genetic differences is difficult to assess in humans. The goal of this study was to determine whether maternal hypercholesterolemia per se may cause enhanced fatty streak formation in offspring and whether interventions during pregnancy can reduce it. During pregnancy, 1 group of New Zealand White rabbits was fed control chow and 8 groups were fed hypercholesterolemic diets Chol 1 (yielding plasma cholesterol of 153 mg/dL) or Chol 2 (yielding 359 mg/dL) without or with cholestyramine, vitamin E, or both. Offspring (n = 15 to 25 per group) were killed at birth. Maternal hypercholesterolemia enhanced mean lesion size in the aorta of their offspring at birth from 44\(\pm\)6\(\times\)10\(^3\) mm\(^2\) per section in controls to 85\(\pm\)6\(\times\)26\(\times\)10\(^3\) mm\(^2\) in Chol 1 and 156\(\pm\)6\(\times\)49\(\times\)10\(^3\) mm\(^2\) in Chol 2 groups (P<0.0001 for both). Cholestyramine or vitamin E treatment of mothers significantly reduced atherosclerosis at birth by up to 39% compared with controls on the same diet. Oxidized fatty acids and malondialdehyde in aortic atherosclerotic lesions and plasma were similarly affected by diets and treatment as atherosclerosis. Our results establish the causal role of hypercholesterolemia and peroxidation in fetal atherogenesis and demonstrate that both lipid-lowering and antioxidant interventions during pregnancy can reduce it. If it can be established that interventions in mothers also affect progression of lesions after birth, this may indicate a novel approach for the prevention of atherosclerosis. (Circ Res. 2000;87:946-952.)

Key Words: atherosclerosis ■ cholestyramine ■ vitamin E ■ oxidation ■ prevention

The atherogenic process in humans already begins in fetuses, and pathogenic events occurring during fetal development profoundly influence the progression of atherosclerosis later in life.\(^1\,^2\) In the past, atherogenesis was thought to begin during late childhood, even though fatty streaks had been observed in younger children.\(^3\,^4\) However, morphometric analysis of cross sections through the aorta of premature human fetuses showed that fatty streaks, the earliest stages of atherosclerotic lesions, already occur in fetal aortas and that their distribution reflects that of more advanced lesions in adults.\(^1\) Remarkably, the number and size of fatty streaks were much greater in fetuses of hypercholesterolemic mothers, including those in whom hypercholesterolemia was limited to pregnancy.

Although animal experiments and human studies had indicated that the placenta is relatively impermeable to large, cholesterol-carrying lipoproteins,\(^5\,^6\) hypercholesterolemia-induced events seemed to be a likely cause of lesion formation in fetuses. Fetal plasma cholesterol levels correlated significantly with maternal ones in fetuses younger than 6 months (but not thereafter). This indicated that maternal hypercholesterolemia itself contributes to enhanced fetal atherogenesis. However, we also observed a striking inverse correlation between fetal age and fetal plasma cholesterol, with very high levels in all younger fetuses (see Reference 1, Figure 3B). Thus, it was conceivable that fetal lesions would regress when cholesterol levels decrease toward the end of pregnancy or under normocholesterolemic conditions after birth.

The Fate of Early Lesions in Children (FELIC) study, designed to investigate the potential influence of fetal lesion formation on later atherogenesis, showed that fetal fatty streaks regress only partially (and not uniformly throughout the aorta) and that maternal hypercholesterolemia was asso-

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We hypothesized that fetal lesion formation and enhanced susceptibility to atherosclerosis later in life were induced, at least in part, by maternal hypercholesterolemia and ensuing enhanced lipid oxidation. A corollary of this hypothesis is that lipid-lowering or antioxidant interventions in mothers during pregnancy may constitute a new approach to reducing atherosclerosis. However, direct experimental evidence for this hypothesis is still outstanding, and differences in the genetic background of normocholesterolemic and hypercholesterolemic mothers may also provide an explanation for both the fetal onset and the enhanced progression of the disease.

We now provide evidence for the causal role of maternal hypercholesterolemia in fetal atherogenesis by showing that in a genetically more homogeneous animal model, the New Zealand White (NZW) rabbit, diet-induced maternal hypercholesterolemia during pregnancy is sufficient to markedly enhance lesions in their offspring at birth. We also demonstrate the involvement of lipid oxidation and establish, in principle, that lipid-lowering and antioxidant interventions during pregnancy are effective in reducing the fetal onset of atherogenesis.

**Materials and Methods**

**Rabbits**

Seventy-seven female NZW rabbits (Harlan-Nossan), age 4.6 ± 1.1 months, were divided into 9 groups (n = 8 to 9) (Table). One group (control) was fed regular chow (4% fat, 18% protein, 60% carbohydrate, and 4% fibers). Four groups each were fed cholesterol-enriched diets Chol 1 or Chol 2 (Teklad adjusted calories diet; 9% fat, 19% protein, 55% carbohydrate, and 4% fibers, to which cholesterol dissolved in ether was added) to achieve total plasma cholesterol (TC) levels of about 150 and 350 mg/dL, respectively (in the absence of lipid-lowering drugs). Diets were started 2 weeks before mating and continued until 2 weeks postpartum. On the basis of preliminary experiments, the cholesterol content of the diet given to the Chol 1 group ranged from 0.132% to 0.156% (depending on each animal’s initial TC level), whereas animals of the Chol 2 group received 0.168% to 0.221% cholesterol. One group (Chol 1) received only the diet. A second group (Chol 1 + CH) received Chol 1 diet plus a dose of cholestyramine (1% to 1.4%) (SIGMA) that was individually adjusted to match the average TC level of the Chow-fed rabbits (~80 mg/dL). A third group (Chol 1 + vitamin E) was fed Chol 1 plus 100 IU/d of vitamin E (α-tocopherol; SIGMA), and a fourth group (Chol 1 + CH + vitamin E) was fed the diet together with both cholestyramine and vitamin E. The four groups fed Chol 2 received the same supplements, except that the dose of cholestyramine (1.8% to 3%) was adjusted to achieve the average TC level of the Chol 1 group. Cholestyramine was chosen as hypcholesterolemic drug because of the virtual absence of side effects during pregnancy. Vitamin E treatment did not result in any adverse effects in mothers or their offspring. The Table provides a summary of experimental conditions. After 2 weeks on the diet, females were bred with untreated males. TC levels were determined at week 2 and 3 of pregnancy by standard enzymatic method. About half of each litter were killed at birth (n = 19 to 25 per group), and the remaining littermates were killed at age 4 months, after about 3 months on regular chow (n = 15 to 18). Experimental groups contained roughly equal numbers of males and females, and data for both genders were analyzed together, because previous studies in human fetuses and children had not indicated significant differences between sexes. All experiments were carried out under approved institutional animal protocols.

**Tissue Preparation and Quantification of Atherosclerosis**

Lesion sizes were determined by computer-assisted image analysis of 30 equidistant frozen oil red O–stained sections each from the aortic arch, thoracic, and abdominal aorta. Additional sections were pooled as lesion or nonlesion tissue. The rationale and methods for tissue preparation and analysis were identical to those previously used for arteries of human fetuses. Results are reported as cumulative lesion area per section (ie, the mean area of all lesions in each of the 90 aortic sections).

**Immunocytochemistry**

Additional sections (n=10) from each aortic segment were formaldehyde fixed and immunostained with 1:500 dilutions of NA59, a...
Results

Maternal plasma cholesterol levels were shown in Figure 1. As expected, Chol 1 and Chol 2 diets raised the average maternal TC levels during pregnancy to 157 and 359 mg/dl, respectively, compared with 58 mg/dl in controls. Cholestyramine, but not vitamin E treatment, significantly reduced TC in both groups to 77 and 171 mg/dL, respectively (P<0.001 vs control; #P<0.001 vs Chol 1; and °P<0.0001 vs same diet+vitamin E. Bonferroni-corrected probability values).

Effect of Hypercholesterolemia and Interventions During Pregnancy on Atherosclerosis in Offspring at Birth

Morphometric assessment of aortic cross sections revealed microscopic fatty streaks even in the control group (Figure 2). Maternal hypercholesterolemia during pregnancy increased lesion sizes by 94% in the Chol 1 group (P<0.0001). Treatment with cholestyramine decreased atherosclerosis by 22% (not significant [NS]), but lesion sizes remained greater than in the control group, even though maternal plasma cholesterol levels of both groups were similar (Figure 1). Offspring of mothers with more marked hypercholesterolemia (Chol 2 group) showed a 253% increase in lesions (P<0.0001) and a powerful antiatherogenic effect of cholestyramine (~30%; P<0.01). Again, lesion sizes in the Chol 2+cholestyramine group significantly exceeded those in the Chol 1 group, which had similar maternal TC levels (Figure 2).
1). Treatment of mothers with vitamin E, which did not lower their cholesterol, significantly reduced atherosclerosis in the Chol 2 group (39%; \( P < 0.0005 \)) but missed significance in the Chol 1 group (219%). Combination of cholestyramine with vitamin E resulted in modest, not significant additional reduction of atherogenesis.

Effect on Plasma Cholesterol and Lipid Oxidation in Offspring at Birth
All groups examined at birth had normal TC levels (48.6 to 58.6 mg/dL; NS) (Figure 3A), indicating that at the end of a regular pregnancy, the placenta is impermeable to maternal LDL and VLDL. Plasma triglycerides were also normal in all groups (32.8 to 42.1 mg/dL; NS). However, plasma concentrations of peroxidative end products, such as 10-OH oleic acid (Figure 3B), 12-OH linoleic acid (Figure 3C), and 10-OH arachidonic acid (data not shown), as well as plasma MDA levels (Figure 3D), were significantly increased in the offspring of mothers fed hypercholesterolemic diets, particularly in those fed Chol 2. Treatment of either diet group with vitamin E significantly reduced peroxidation (Figures 3B through 3D), whereas cholestyramine caused only a much smaller reduction of oxidation.

Effect on Aortic Lipid Oxidation and Lesion Composition at Birth
Concentrations of oxidized oleic and linoleic acids (Figures 4A and 4B) and arachidonic acid (not shown) in early atherosclerotic lesions of the aorta were markedly elevated in rabbits of the Chol 1 and Chol 2 groups. Maternal treatment with vitamin E was associated with a highly significant reduction of oxidized FAs in the Chol 2 group. A reduction was also seen in the Chol 1 group, but this was significant only for linoleic acid. As in plasma, offspring of cholestyramine-treated mothers also showed significant reductions in oxidized FAs in early aortic lesions, and no additional significant reduction was achieved by combination therapy. Immunocytochemical detection of typical components of early lesions, ie, oxidation-specific epitopes such as 4-hydroxynonenal-lysines detected by NA59 (Figure 4C) and oxidized phospholipid epitopes detected by EO6 (Figure 4D), as well as LDL (apolipoprotein B) (Figure 4E), and macrophages and foam cells (Figure 4F), showed similar protective effects of antioxidant and lipid-lowering interventions during pregnancy. As expected, aortic concentrations of nonoxidized linoleic, oleic, and arachidonic acids were also increased by hypercholesterolemic diets (data not shown).

Correlation Between Maternal Plasma Cholesterol Levels and Atherosclerosis at Birth
The above results suggested that the marked increase in atherosclerosis in the Chol 1 and Chol 2 groups was a direct result of maternal hypercholesterolemia. Indeed, linear regression analysis of all data indicated a correlation between the average maternal plasma cholesterol level during pregnancy and the size of lesions in their offspring at birth (Figure 5). Data of the cholestyramine-treated groups also were
consistent with the assumption that the reduction of lesions was mainly attributable to the reduction of TC. However, only 37% of atherosclerosis could be explained by the maternal cholesterol levels. Furthermore, treatment with vitamin E was associated with a marked reduction in lesion size (Figures 2 and 5) but did not affect cholesterol levels. When data from groups receiving vitamin E were excluded, the correlation coefficient increased to \( r = 0.78 \). The size of atherosclerotic lesions also significantly correlated with maternal plasma MDA, particularly when only groups not receiving vitamin E were analyzed \( (r = 0.62, P < 0.0001) \).

Effects of Hypercholesterolemia and Interventions During Pregnancy at Age 4 Months

To detect whether fetal lesions progress in the absence of postnatal hypercholesterolemia, some of the offspring were fed regular chow and examined at age 4 months (Figure 6). Atherogenesis in the Chol 1 and Chol 2 groups was greater than in the chow group by 224% and 323%, respectively. However, absolute lesion sizes in all groups tended to be smaller than those in their siblings at birth, even though their aortas were much larger. This was particularly the case in the control, Chol 2, and Chol 2 + CH groups \( (P < 0.0001) \). Thus, lesions formed during fetal development regress, rather than progress, under the extremely low cholesterol levels found in chow-fed NZW rabbits \( (49 \pm 2.1 \text{ to } 58 \pm 1.6 \text{ mg/dL}; \text{ NS}) \). As expected, plasma concentrations of peroxidative end products at 4 months were much lower than at birth in absolute terms, and significant decreases in treatment groups were only seen for 10-OH oelic acid and MDA and only in the Chol 2 groups (data not shown). In contrast, concentrations of peroxidative end products in aortic lesions were only slightly lower than those at birth (NS). Significant treatment effects persisted for 10-OH oelic acid \( (eg, P < 0.05 \text{ in all Chol 1 treatment groups}) \) and oxidation-specific epitopes \( (eg, P < 0.001 \text{ to } P < 0.01 \text{ in all Chol 1 treatment groups for EO6-positive sections}) \), whereas aortic levels of nonoxidized FAs were similar in all groups.

An additional experiment following the same protocol showed that lesions and some differences between treatment groups still persisted at age 7 months. But, again, this did not indicate progression (data not shown).

Discussion

We previously showed that atherogenesis in human offspring of hypercholesterolemic mothers was more extensive at fetal age and progressed faster during childhood and adolescence.\(^3\) The present study investigated the mechanisms responsible for the formation of early lesions during pregnancy using an inbred rabbit model, in which genetic differences are far less prevalent than in humans. It provides two main findings. The first is that temporary induction of maternal hypercholesterolemia in NZW rabbits by dietary intervention enhances fatty streak formation in their offspring. This demonstrates that increased early lesion formation similar to that seen in human fetuses can be caused by events associated with maternal hypercholesterolemia and does not necessarily require genetic differences that are likely to exist between normocholesterolemic and hypercholesterolemic human mothers (and fathers). The fact that cholestyramine lowered cholesterol levels in mothers during pregnancy and decreased lesions in their offspring strongly supports a causal role of maternal hypercholesterolemia in fetal atherogenesis. The second main finding is that intervention with vitamin E during pregnancy also markedly reduced fetal lesion sizes. This provides evidence for the causal role of processes influenced by lipid oxidation in fetal atherogenesis. Together, these data demonstrate, in principle, that hypolipidemic and antioxidant interventions during pregnancy may constitute a novel approach to prevention of atherosclerosis.

In our study, maternal plasma cholesterol in the Chol 1 and Chol 2 groups was raised from a normal level of 58 mg/dL to 153 and 359 mg/dL, respectively (Figure 1). Both dietary and plasma cholesterol concentrations were much lower than those conventionally used to induce atherosclerosis in NZW rabbits. Therefore, it is not surprising that the average lesion area in the aorta at birth was very small in absolute terms. Our experimental conditions were designed to achieve cholesterol levels comparable to those of human mothers in the hypercholesterolemic groups of our previous studies \( (280 \text{ and } 460\)
Maternal hypercholesterolemia of NZW rabbits resulted in marked increases in aortic lesion sizes in their offspring at term birth, which was greatest in the group exposed to the highest cholesterol levels (Chol 2) (Figure 2). Cholestyramine consistently reduced both maternal cholesterol and lesion sizes in offspring, indicating a direct correlation between the two parameters. Linear regression analysis of all groups not receiving antioxidants confirmed a substantial influence of the maternal cholesterol on lesion sizes (Figure 5). However, the extent of atherosclerosis in the cholestyramine groups was not quite reduced to that of untreated groups with matching cholesterol levels (Figures 1 and 4), indicating involvement of atherogenic mechanisms other than hypercholesterolemia. Although maternal hypercholesterolemia thus plays a major role in fetal atherogenesis, it remains unclear whether this means an increased transplacental passage of maternal LDL and VLDL, increased transfer of FAs, or increase passage of oxidized FAs, oxidized FA fragments, or other transducers or end effectors.

One of the potential mechanisms contributing to the atherogenic effect of maternal hypercholesterolemia is an increase of peroxidative end products in both mother and fetus. LDL oxidation not only occurs in atherosclerotic lesions but also in fatty streaks of human fetuses. Several pathways by which OxLDL may promote atherogenesis have been identified. These include accelerated foam cell formation by enhanced macrophage uptake of OxLDL via scavenger receptors and the recruitment of monocytes and T-cells into the intima. Oxidation also influences nuclear receptor pathways, such as nuclear factor-κB and peroxisome proliferator-activated receptor γ, which regulate expression of adhesion molecules, growth factors, and proinflammatory genes, and affect cell cycle and apoptosis. Regulation of vasotocin by nitric oxide is also impaired by OxLDL. Finally, OxLDL has profound immunological consequences that modulate atherogenesis. Extensive evidence indicates that hypercholesterolemia is accompanied by increased plasma levels of peroxidative products. (Conversely, in our study the cholestyramine-induced reduction of maternal TC was asso-
ciated with a decrease of peroxidative end products in plasma and, more importantly, in early atherosclerotic lesions.) We had postulated, therefore, that oxidation-sensitive mechanisms play an important role in lesion formation associated with maternal hypercholesterolemia.

Antioxidant intervention during pregnancy markedly reduced early atherogenesis. The fact that this was not associated with a decrease in plasma cholesterol strongly supports the pathogenic role of oxidation, but we cannot rule out unrelated effects of vitamin E in the arterial wall. As expected, peroxidative end products, such as oxidized FAs and MDA, were markedly reduced in early lesions and plasma of vitamin E–treated groups. Cholestyramine also reduced lipid oxidation, albeit to a lesser extent, suggesting that substrate availability is a major determinant. However, shifts in placental passage of specific FAs resulting from the hypocholesterolemic intervention may also have played a role. Combinations of cholestyramine and vitamin E tended to be even more protective than cholestyramine or vitamin E alone, in particular in rabbits fed Chol 2, but failed to completely abolish the increase in lesion formation compared with normocholesterolemic controls, probably because of insufficient protection achieved under the present experimental conditions.

The present results demonstrate the role of maternal hypercholesterolemia in early atherogenesis in offspring and the efficacy of interventions with vitamin E or cholestyramine. The results also establish an experimental model of human fetal atherogenesis, but additional studies are needed to determine whether fetal hypercholesterolemia and lipid peroxidation also accelerate the progression of atherosclerosis and whether interventions in mothers affect this. Our first exploratory experiment indicated that fetal lesions and therapeutic effects persist for a prolonged time but could not test its long-term consequences in the absence of lesion progression. The absence of atherogenesis or regression of fetal lesions is probably attributable to the fact that cholesterol levels in chow-fed NZW rabbits are extremely low compared with what is considered normal in humans. This may be overcome in future experiments by exposing rabbits to a mildly hypercholesterolemic diet after birth. Nevertheless, the lack of progression suggests that other risk factors of atherosclerosis, including differences in genetic background, may be required for the faster progression of atherosclerosis seen in children of hypercholesterolemic mothers.

If it can be established that fetal lesion formation influences the rate of progression of atherosclerosis later in life, our results would indicate novel approaches for prevention. Dietary intervention in pregnant women may be one way to lower cholesterol. Cholestyramine also seems to be safe during pregnancy and is commonly used in pruritus associated with cholestasis. Antioxidant interventions significantly reduced atherosclerosis in experimental models but had only mixed success in preventing recurrent cardiovascular events in patients with preexisting coronary heart disease. However, it is possible that antioxidant protection is most effective in early stages of atherosclerosis and, therefore, may offer greater benefits to fetuses than to adults. We chose vitamin E and cholestyramine because of their safety during pregnancy and to prove, in principle, that lipid-lowering and antioxidant intervention may be beneficial, but more powerful hypolipidemic drugs and antioxidants may offer greater benefits. Finally, by demonstrating the role of maternal hypercholesterolemia, our results support the inclusion of maternal hypercholesterolemia during pregnancy among the risk factors that determine the need for more intense monitoring in children than currently recommended.

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