Eliminating Atherogenesis in Mice by Switching Off Hepatic Lipoprotein Secretion

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Background—LDL receptor–deficient “apolipoprotein (apo)-B100–only” mice (Ldlr\textsuperscript{−/−} Apob\textsuperscript{100/100}) have elevated LDL cholesterol levels on a chow diet and develop severe aortic atherosclerosis. We hypothesized that both the hypercholesterolemia and the susceptibility to atherosclerosis could be eliminated by switching off hepatic lipoprotein production.

Methods and Results—We bred Ldlr\textsuperscript{−/−} Apob\textsuperscript{100/100} mice that were homozygous for a conditional allele for Mttp (the gene for microsomal triglyceride transfer protein) and the inducible Mx1-Cre transgene. In these animals, which we called “Reversa mice,” the hypercholesterolemia could be reversed, without modifying the diet or initiating a hypolipidemic drug, by the transient induction of Cre expression in the liver. After Cre induction, hepatic Mttp expression was virtually eliminated (as judged by quantitative real-time PCR), hepatic lipoprotein secretion was abolished (as judged by electron microscopy), and LDLs were virtually eliminated from the plasma. Intestinal lipoprotein production was unaffected. In mice fed a chow diet, Cre induction reduced plasma cholesterol levels from 233.9±46.0 to 37.2±6.5 mg/dL. In mice fed a high-fat diet, cholesterol levels fell from 525.7±32.2 to 100.6±14.3 mg/dL. The elimination of hepatic lipoprotein production completely prevented both the development of atherosclerosis and the changes in gene expression that accompany atherogenesis.

Conclusions—We developed mice in which hypercholesterolemia can be reversed with a genetic switch. These mice will be useful for understanding gene-expression changes that accompany the reversal of hypercholesterolemia and atherosclerosis. (Circulation. 2003;107:1315-1321.)

Key Words: cholesterol ■ hypercholesterolemia ■ apolipoproteins ■ lipoproteins ■ atherosclerosis

Hypercholesterolemic mice are widely used in atherosclerosis research. Apolipoprotein (apo) E–deficient (Apoe\textsuperscript{−/−}) mice have elevated plasma concentrations of apo-B48—containing remnant lipoproteins and develop advanced atherosclerosis by 4 to 6 months of age, even when fed a low-fat diet.\textsuperscript{1,2} LDL receptor–deficient (Ldlr\textsuperscript{−/−}) mice have elevated levels of apo-B100—containing LDL in their plasma. However, Ldlr\textsuperscript{−/−} mice have far lower total plasma cholesterol levels than Apoe\textsuperscript{−/−} mice on a chow diet and develop only tiny atherosclerotic lesions.\textsuperscript{3} We recently demonstrated that “apo-B100–only” LDL receptor–deficient mice (Ldlr\textsuperscript{−/−} Apob\textsuperscript{100/100}) have higher LDL cholesterol levels than Ldlr\textsuperscript{−/−} mice on a chow diet and develop severe atherosclerosis—even more severe than that of Apoe\textsuperscript{−/−} mice.\textsuperscript{5}

The hypercholesterolemic mouse models have been a boon for atherosclerosis research. Genetic studies with these mice have made it possible to define the impact of many genes on the development of atherosclerosis\textsuperscript{6–9} and also to document the gene-expression changes that accompany atherogenesis.\textsuperscript{10} However, experimental approaches with these mouse models are limited. For example, if one were interested in inducing the regression of atherosclerosis or in defining the effects of plasma cholesterol lowering on gene expression in the arterial wall, the existing mouse models would be of little use. Ldlr\textsuperscript{−/−} and Apoe\textsuperscript{−/−} mice remain hyperlipidemic after virtually all of the fat and cholesterol is removed from the diet,\textsuperscript{9} and neither animal model responds particularly well to the cholesterol-lowering effects of statins.\textsuperscript{11,12} Severe atherosclerotic lesions in Apoe\textsuperscript{−/−} mice can be induced by feeding them a high-fat diet,\textsuperscript{1,2,13} but switching the mice to a low-fat chow diet does not cause lesion regression,\textsuperscript{14} because plasma lipid levels remain quite high.\textsuperscript{9}
We reasoned that it would be useful to develop a hypercholesterolemic mouse in which the elevated levels of cholesterol in the plasma could be reversed with a simple genetic intervention. A potential strategy was suggested by a recent study in which Cre/loxP recombination techniques were used to switch off a conditional allele of microsomal triglyceride transfer protein (Mttp) in the liver, thereby preventing hepatic lipoprotein assembly and secretion. We predicted that it might be possible to produce mice with “reversible hypercholesterolemia” by breeding Ldlr<sup>−/−</sup>Apo<sub>100/100</sub> mice that also were homozygous for a conditional Mttp allele and an inducible Cre transgene. In such an animal, we hypothesized that the induction of Cre would eliminate LDL from the plasma and that the resultant fall in cholesterol levels would reduce susceptibility to atherogenesis. We further hypothesized that the reduction in plasma cholesterol levels would abrogate the arterial wall gene-expression perturbations that accompany atherogenesis. In the present studies, we bred Ldlr<sup>−/−</sup>Apo<sub>100/100</sub> mice with the conditional Mttp allele and the Cre transgene and tested each of those hypotheses.

**Methods**

**Genetically Modified Mice**

Ldlr<sup>−/−</sup> mice<sup>1</sup> homozygous for an apo-B100–only alleles<sup>17</sup> were bred with mice that were homozygous for a conditional (“flanked”) Mttp allele<sup>15</sup> and the Mx1-Cre transgene<sup>18</sup> (Mttp<sub>100/100</sub>Mx1-Cre<sup>−/−</sup>). Offspring were bred to produce mice that were homozygous for all 4 alleles (Ldlr<sup>−/−</sup>Apo<sub>100/100</sub>Mttp<sub>100/100</sub>Mx1-Cre<sup>−/−</sup>). Those mice, called “Reversa mice,” were intercrossed for 3 generations. All mice were bred in our lab, weaned at 28 days of age, and fed a chow diet containing 4.5% fat (Ralston Purina) or a high-fat diet containing 20% fat (Dyets). A Student t-test was used to compare lipid levels before and after treatment. By fast-performance liquid chromatography (FPLC) on a Superose 6 10/30 column.4,5 Mice were fasted for 4 hours before blood sampling, except when indicated otherwise.

**Ultrastructural Analysis of Lipoprotein Assembly in Mouse Liver**

Tissue samples were embedded in epoxy resin after perfusion fixation with 1.5% glutaraldehyde, 4% polyvinylpyrrolidone (molecular weight 10 000), and 0.05% calcium chloride in 100 mmol/L sodium cacodylate buffer (pH 7.4). Electron microscopy was performed on liver tissue that had been stained with the imidazole-buffered osmium tetroxide procedure.13,20,21

**Analysis of Lipoprotein Particle Size**

The d<sub>1.052</sub>-fraction of plasma was prepared by ultracentrifugation, and lipoprotein particle diameters were determined by dynamic light scattering analysis with a Microtrac Series 150 Ultrafine particle analyzer fitted with a flexible conduit-sheathed probe tip (UPA-150; Microtrac).<sup>3</sup> Data were plotted as the percentage of total particles in different particle size ranges.

**Analysis of Mouse Atherosclerosis**

Reversa mice were placed on a high-fat diet at 4 weeks of age. One group (n=6) was immediately treated with pL-pC, and the other group (n=8) was untreated. After 20 weeks on the high-fat diet, both groups of mice were euthanized. Mice were perfused with PBS followed by a fixative solution (4% paraformaldehyde, 5% sucrose, 20 mmol/L EDTA; pH 7.4). With the major branching vessels attached, the aorta was opened longitudinally from the iliac arteries to the aortic root. Then, all branching vessels were removed, and the aorta was pinned out flat on a black wax surface. Images of the pinned-out aortas were recorded with a Polaroid digital microscope camera. Each image was analyzed with Adobe Photoshop 5.5 (Adobe Systems) with gray and white threshold to define lesions, and the percentage of the surface covered by lesions was calculated.<sup>8</sup> Lesions were quantified by a trained observer blinded to treatment group.

**Analysis of Arterial Gene Expression**

Reversa mice [either nontreated (n=5) or pL-pC–treated (n=4)] were weaned onto a high-fat diet. After 20 weeks, the mice were perfused with PBS, and the thoracic aorta was removed. The thoracic aorta was then separated into 2 segments (the aortic arch and the lower thoracic aorta) by cutting the aorta 3 mm distal to the origin of the left subclavian artery. RNA from the pooled aortic tissues was prepared with the RNeasy kit (Qiagen). To eliminate the possibility of DNA contamination, each RNA sample was treated with DNase (2.0 U, DNA-free, Ambion) in the presence of RNase inhibitor (RNaseOut, Invitrogen Life Technologies). Reverse transcription of RNA was performed with an oligo(dT), and the expression levels of CD68, vascular cell adhesion molecule-1 (VCAM-1), monocyte chemoattractant protein-1 (MCP-1), and intercellular adhesion molecule-1 (ICAM-1) were determined by quantitative real-time PCR on an ABI Prism 7700 Sequence Detection System (Applied Biosystems), as previously described.<sup>10</sup> Expression levels were normalized to the expression of cyclophilin A.<sup>10</sup>

**Statistical Analysis**

Data are expressed as mean±SEM. Measurements were analyzed with Primer of Biostatistics Software (Version 3.0, McGraw Hill, 1992). A Student’s 2-tailed t test was used to compare lipid levels before and after treatment.

**Lipid Measurements and Lipoprotein Analyses**

Total plasma cholesterol and triglyceride concentrations were measured on fresh plasma with colorimetric assays (Spectrum Cholesterol Assay; Abbot Laboratories; and Triglyceride/GB Kit; Roche). The distribution of lipids within the plasma lipoproteins was determined by fractionating mouse plasma (350 μL pooled from 10 mice) by fast-performance liquid chromatography (FPLC) on a Supersose 6 10/30 column.<sup>3,5</sup> Mice were fasted for 4 hours before blood sampling, except when indicated otherwise.

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Results

Inactivating Mtp in Reversa Mice With pl-pC

Treatment of Reversa mice with pl-pC was effective in inducing recombination within the Mtp in the liver. Southern blotting revealed that the vast majority of liver genomic DNA had undergone recombination, converting the Mtp<sup>a</sup> allele to a Mtp<sup>o</sup> allele (Figure 1A). Small amounts of recombination were observed in tail DNA. The pl-pC treatment nearly abolished Mtp mRNA levels, as judged by quantitative real-time PCR (Figure 1B), and Western blotting revealed a striking reduction in plasma apo-B100 levels (Figure 1C). The presence of some apo-B100 in the plasma of the pl-pC–treated mice was expected. Apo-B100 is produced by the intestines of Reversa mice, and the extent of Mtp inactivation in that tissue is minimal. 15

Ultrastructural examination of liver samples revealed that VLDL production was abolished in the pl-pC–treated Reversa mice (Figure 2). Hepatocytes from untreated Reversa mice (Figure 2A) contained numerous lipoprotein particles in the Golgi stacks and vesicles. In the pl-pC–treated mice, VLDL particles were absent, although there were a few small, irregularly shaped lipid-staining particles in the Golgi stacks (Figure 2B). Of note, the inactivation of Mtp was virtually complete in Reversa mice. By electron microscopy, we could not identify any VLDL-producing hepatocytes in pl-pC–treated mice. Light-level histological studies showed an increased amount of neutral lipids in the livers of the pl-pC–treated mice (not shown).

Plasma Lipid Levels in Reversa Mice Fall After Induction of Cre Expression

In mice on a chow diet, the plasma cholesterol levels fell from 233.9±46 to 37.2±6.5 mg/dL (n=15; P<0.001) after pl-pC treatment (Figure 3A), and the triglycerides fell from 70.2±7.88 to 16.3±8.4 mg/dL (n=12; P<0.001) (Figure 3B). In mice on the high-fat diet, plasma cholesterol levels fell from 525.7±32.24 to 100.6±14.33 mg/dL (n=16; P<0.001) (Figure 3A), and triglyceride levels fell from 110.33±6.45 to 23.4±4.56 mg/dL (n=14; P<0.001) (Figure 3B). The plasma lipid levels reached their nadirs ~8 to 10 days after the first pl-pC injection (Figure 3, C and D).

The distribution of lipids within the plasma lipoproteins was assessed by FPLC fractionation studies (Figure 4). In the plasma of chow-fed Reversa mice, there was a large IDL/LDL cholesterol peak. This peak was virtually eliminated by pl-pC treatment (Figure 4A). On the high-fat diet, cholesterol levels were higher, but the findings were similar (Figure 4B). On both diets, HDL cholesterol levels were lower in the pl-pC–treated mice. The pl-pC treatment caused a striking reduction in the triglyceride contents of VLDL, IDL, and LDL on both diets (Figure 4, C and D).

Spectrum of Lipoprotein Particle Size Shifts After Induction of Cre Expression

In chow-fed mice under nonfasting conditions, most of the lipoproteins in the plasma of the Reversa mice were in the LDL size range (~20 nm) (Figure 5). The same was the case for Ldlr<sup>−/−</sup> Apob<sup>100/100</sup> controls. After treatment of the Reversa mice with pl-pC, the lipoproteins that remained in nonfasting plasma had a bimodal size distribution. A sizable percentage of the particles were immense, with diameters >150 nm. A second peak in the particle size distribution occurred at ~33 nm. We suspect that the larger particles represented nascent

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Figure 1. A, Southern blot analysis of Cre-mediated recombination within Mtp in liver. Southern blot strategy differentiates between wild-type Mtp allele (Mtp<sup>+</sup>, 16.5 kb), conditional Mtp allele (Mtp<sup>+</sup>, 8 kb), and inactivated Mtp allele (Mtp<sup>+</sup>, 11.5 kb). B, Hepatic Mtp expression levels in Reversa mice as judged by quantitative real-time PCR (normalized to Gapdh). C, Western blot analysis of relative amounts of apo-B100 in plasma of untreated and pl-pC–treated Reversa mice. Plasma from an Apob<sup>48/48</sup> mouse was included as a control. Inactivation of Mtp was less complete in mice with a single copy of Mx1-Cre transgene (not shown).
chylomicrons, because that peak was completely absent after an overnight fast (not shown). The smaller particles, which at \( \approx 33 \) nm were significantly larger than LDL, were probably chylomicron remnants. We strongly suspect that the chylomicron particles were also present in the plasma of untreated Reversa mice (no pI-pC). However, the large particles represented a very tiny percentage of the total particles in those animals and therefore did not appear on the particle distribution plot. In the pl-pC–treated mice, the elimination of hepatic lipoproteins unmasked the presence of the very large intestinal lipoproteins.

**Prevention of Atherosclerosis by Induction of Cre Expression**
To determine whether Cre-mediated inactivation of Mttp prevents atherosclerosis, Reversa mice \((n=6)\) were treated at 4 weeks of age with pl-pC and then placed on a high-fat diet. Littermate controls \((n=8)\) did not receive pl-pC. After 20 weeks, atherosclerotic lesions covered 5.2\(\pm\)0.8\% of the surface of the aorta in the untreated mice. Lesions were prominent in the aortic arch, but only tiny lesions were present in the remainder of the aorta, primarily at vessel branch points (Figure 6A). In contrast, pl-pC–treated mice had no detectable lesions (Figure 6B).

**Arterial Wall Gene Expression in Reversa Mice**
We suspected that untreated Reversa mice (no pl-pC) would have greater expression of CD68 (a macrophage marker) in the aorta than the pl-pC–treated mice. We also suspected that the untreated mice would have higher mRNA levels for VCAM-1, MCP-1, and ICAM-1. Indeed, this proved to be the case (Figure 7). Expression levels of CD68, ICAM-1, and MCP-1 in the aortic arch were much higher in untreated mice than in pl-pC–treated mice. CD68, VCAM-1, and MCP-1 expression levels were much lower in the lower thoracic aorta, which contained far fewer lesions. ICAM-1 expression in the aortic arch was also greater in untreated mice than in pl-pC–treated mice (Figure 7), although the relative difference was less than with the other genes. ICAM-1 expression seems to be more dependent on shear stress than on plasma lipid levels.22

**Discussion**
In this study, we produced Reversa mice, a hypercholesterolemic mouse model in which high plasma cholesterol levels...
can be eliminated, without hypolipidemic drugs or changing the diet, by flipping a genetic switch. At baseline, Reversa mice had high levels of LDL in their plasma, very similar to the lipoprotein profile of most humans with coronary artery disease. After induction of Mx1-Cre expression with pI-pC, hepatic Mttp mRNA levels fell precipitously, lipoprotein production in the liver was abolished, and LDL cholesterol in the plasma was virtually eliminated. The striking change in LDL cholesterol levels can be achieved without affecting the overall nutritional status of the mice. The pI-pC injections do not cause significant levels of recombination in intestinal enterocytes. After pI-pC, Reversa mice appeared to be healthy and exhibited normal levels of vitality. They consumed normal amounts of food (H. Lieu, unpublished observations) and had very large chylomicrons in their plasma.

In an earlier study, we demonstrated that Ldlr−/− Apob100/100 mice have high LDL cholesterol levels and develop severe atherosclerosis after 40 weeks on a chow diet, with 10% to 15% of the surface of the aorta covered with sudanophilic lesions. In the present study, we sought to determine whether the blockade of hepatic lipoprotein secretion would eliminate the heightened susceptibility of those mice to atherosclerosis. We quantified atherosclerosis in pI-pC–treated Reversa mice...
eride-rich lipoproteins to nascent apo-AI lipids (phospholipids and free cholesterol) from the triglyceride. 24 It is conceivable that the low HDL cholesterol levels in the untreated Reversa mice could be in part a result of the change in plasma cholesterol levels in Reversa mice is so striking, we suspect that this model will make it possible to study atherosclerosis regression in the mouse. We would be surprised if the elimination of LDL cholesterol did not cause regression of atherosclerotic lesions, as judged either by Sudan IV staining or by the quantification of cholesteryl esters in aortic lesions. 5 Perhaps more interesting, however, will be to determine whether and to what extent the elimination of plasma LDL cholesterol causes the disappearance of free cholesterol crystals, macrophages, and the fibrous tissue within lesions. Second, because plasma cholesterol levels in Reversa mice can be titrated (by adjusting the dose of pl-pC), it will be possible to design better control groups for certain types of atherosclerosis experiments, making it possible to sharpen certain experimental conclusions. For example, in assessing the impact of certain antioxidants or certain dietary fatty acids on atherogenesis, it has occasionally been difficult to distinguish between the direct effects of the antioxidants or fatty acids on the development of lesions from the indirect effects of those agents on plasma cholesterol levels. If one were to perform those experiments in Reversa mice, it would be possible to titrate pl-pC dose so as to obtain groups of experimental animals that were matched for plasma cholesterol levels.

In the present study, we demonstrate that inhibition of hepatic lipoprotein secretion lowered the expression of several genes in the arterial wall that are associated with atherogenesis. The expression of CD68, VCAM-1, and MCP-1 in aortic tissue was strikingly lower in pl-pC–treated Reversa mice than in untreated controls. In part, this difference undoubtedly reflects different numbers of macrophages within the arterial wall tissue. However, the higher expression levels in the untreated Reversa mice could be in part a result of activation of the macrophages by the high levels of apo-B100–containing lipoproteins (and oxidized lipoproteins 25 ) within the arterial wall. In a recent publication, Trogan et al 10 used laser-capture microdissection techniques to demonstrate that the expression of CD68, VCAM-1, and MCP-1 in arterial wall macrophages was increased by activating macrophages with bacterial lipopolysaccharides. It would be interesting to use the same experimental techniques to determine whether activated macrophages in atherosclerotic lesions could be deactivated by lowering plasma LDL cholesterol levels. If so, would the macrophage deactivation require a threshold level of cholesterol lowering? Also, would the macrophage deactivation occur immediately or only several months later? The existence of Reversa mice now makes it practical to answer these questions.

Reversa mice can now be ordered from The Jackson Laboratory (http://jaxmice.jax.org/jaxmicedb/html/gemm_target.shtml).

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