Increased Low-Density Lipoprotein Oxidation and Impaired High-Density Lipoprotein Antioxidant Defense Are Associated With Increased Macrophage Homing and Atherosclerosis in Dyslipidemic Obese Mice

LCAT Gene Transfer Decreases Atherosclerosis

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Background—Obesity-associated dyslipidemia in humans is associated with increased low-density lipoprotein (LDL) oxidation. Mice with combined leptin and LDL receptor deficiency are obese and show severe dyslipidemia and insulin resistance. We investigated the association between oxidation of apolipoprotein B–containing lipoproteins, high-density lipoprotein (HDL) antioxidant defense, and atherosclerosis in these mice.

Methods and Results—LDL receptor knockout (LDLR<sup>−/−</sup>), leptin-deficient (ob/ob), double-mutant (LDLR<sup>−/−</sup>;ob/ob), and C57BL6 mice were fed standard chow. Double-mutant mice had higher levels of non-HDL (P<0.001) and HDL (P<0.01) cholesterol and of triglycerides (P<0.001). They also had higher oxidative stress, evidenced by higher titers of autoantibodies against malondialdehyde-modified LDL (P<0.001). C57BL6 and ob/ob mice had no detectable lesions. Lesions covered 20% of total area of the thoracic abdominal aorta in double-mutant mice compared with 3.5% in LDLR<sup>−/−</sup> mice (P<0.01). Higher macrophage homing and accumulation of oxidized apolipoprotein B-100–containing lipoproteins were associated with larger plaque volumes in the aortic root of double-mutant mice (P<0.01).

The activity of the HDL-associated antioxidant enzymes paraoxonase and lecithin:cholesterol acyltransferase (LCAT) (ANOVA; P<0.0001 for both) was lower in double-mutant mice. Adenovirus-mediated LCAT gene transfer in double-mutant mice increased plasma LCAT activity by 64% (P<0.01) and reduced the titer of autoantibodies by 40% (P<0.01) and plaque volume in the aortic root by 42% (P<0.05) at 6 weeks.

Conclusions—Dyslipidemia and insulin resistance in obese LDL receptor–deficient mice are associated with increased oxidative stress and impaired HDL-associated antioxidant defense, evidenced by decreased paraoxonase and LCAT activity. Transient LCAT overexpression was associated with a reduction of oxidative stress and atherosclerosis. (Circulation. 2003;107:1640-1646.)

Key Words: atherosclerosis • obesity • hypercholesterolemia • lipoproteins
but did not change cholesterol production in ob/ob mice regardless of their LDLR genotype.

We have used this model to additionally study the mechanisms underlying atherosclerosis in these obese dyslipidemic mice. We have investigated the effect of hypercholesterolemia and insulin resistance on LDL oxidation because we have demonstrated increased LDL oxidation in obese subjects that are at high risk for cardiovascular disease.6 We have particularly investigated the cholesterol acceptor and antioxidant function of high-density lipoprotein (HDL).7 It is well-known that HDL protects against cardiovascular disease. The HDL-associated enzymes paraoxonase (PON) and lecithin:cholesterol acyltransferase (LCAT) inhibit the oxidation of LDL.10,11 Platelet activating factor-acetylhydrolase (PAF-AH) degrades bioactive oxidized phospholipids,12 PON and PAF-AH both actively protect hypercholesterolemic mice against atherosclerosis.13,14 We demonstrate that increased LDL oxidation and macrophage infiltration, in association with decreased HDL-associated paraoxonase and LCAT activity, is associated with accelerated atherosclerosis in obese dyslipidemic mice. Transient overexpression of LCAT reduced oxidation of LDL, macrophage accumulation, and atherosclerosis.

Methods

Animal Experiments

Homozygous LDL receptor knockout mice (LDLR−/−), heterozygous ob/+/+, and C57BL6 mice were purchased from Jackson Laboratory (Bar Harbor, Maine). LDLR−/− mice were backcrossed into a C57BL6 background to the tenth generation and had 98.4% C57BL6 background. To obtain leptin deficiency (ob/ob) on a background of LDLR deficiency, LDLR−/+ and ob/+ mice were crossed, and the F1 progeny of this mating (LDLR−/+ob/+ ) were then crossed to obtain mice that had either zero, one, or both normal LDLR alleles and were leptin-deficient (LDLR−/−ob/ob, LDLR−/+ob/ob, and LDLR−/− ob/ob, respectively) as well as control LDLR−/+ , LDLR−/−, and wild-type mice. All offspring were genotyped by polymerase chain reaction (PCR) techniques as previously described.6 All mice were housed at 22°C on a fixed 14/10-hour light/dark cycle and were fed standard chow. All experimental procedures in animals were performed in accordance with protocols approved by the Institutional Animal Care and Research Advisory Committee.

Blood Analysis

Blood from 22- to 26-week-old mice was collected into EDTA tubes. Plasma was obtained by centrifugation, and lipoproteins were separated by FPLC.13 Phospholipid levels were determined with an enzymatic assay (Biomerieux). Protein levels were determined according to Bradford.16 free cholesterol and cholesterol ester levels by high-performance liquid chromatography,14 triglyceride levels with a diagnostic reagent kit (Sigma-Aldrich), glucose levels with a glucometer (Menarini Diagnostics), and plasma insulin levels with a mouse insulin ELISA (Mercodia). The titers of Ig autoantibodies against malondialdehyde (MDA)-modified LDL were determined in individual plasma samples (1:500 dilution). The amount of Ig bound to the MDA-LDL antigen was detected with alkaline phosphatase-labeled anti-mouse IgG. Data are expressed as relative absorbance units.18

In Vitro Cellular Cholesterol Efflux

J774 cells were used to compare the abilities of HDL samples to induce efflux of cellular cholesterol, as described before.17,19

In Vivo Macrophage Homing and Atherosclerosis

In vivo macrophage homing was assessed as previously described7,20 using fluorescein-labeled C57BL6 peritoneal macrophages. The extent of atherosclerosis in the thoracic abdominal aorta was measured en face using methods described previously.17,19 Cholesterol deposits within the aorta were visualized by staining with Sudan IV, and the extent of lesion was expressed as percentage of aorta covered by lesion.21 For measuring atherosclerosis in the aortic root, hearts and aortas were fixed in 4% phosphate-buffered formaldehyde and embedded in 25% gelatin. Approximately 12 7-μm frozen sections were used for morphometric and immunohistochemical analysis.17 Lipids were stained with oil-red O, oxidized LDL with mAb4E6, smooth muscle cells with an α-actin–specific antibody (Dako), macrophages with an antibody against mouse Mac-3 antigen (Pharmingen), and myeloperoxidase with an antibody against human myeloperoxidase (Dako). Blinded analysis of positive immunostained sections was performed with the Quantimet600 image analyzer (Leica).14 A color intensity threshold mask for immunostaining was defined to detect the red color by sampling, and the same threshold was applied to all specimens. The percentage of the total area with positive color for each section was recorded.22

PAF-AH, PON Activity, and LCAT Activity

PAF-AH and PON activity was measured as previously described23,24 for mouse plasma. LCAT activity was measured using an exogenous proteoliposome substrate containing [4Cl]cholesterol10

LCAT Gene Transfer

Human LCAT cDNA was a gift from Genentech (San Francisco, Calif). It was subcloned in the shuttle plasmid pAcpPla downstream of the ApoAl promoter and upstream of 4 copies of ApoE enhancer, as previously described.25 The control recombinant adenovirus AdRR5 has been described elsewhere.26 Plaque-forming units 5 × 104 of AdLCAT or AdRR5 virus were injected into the tail vein of LDLR−/− or ob/ob mice.

RNA Extraction and Real-Time RT-PCR

Total RNA was extracted from mouse aortas, and first-strand cDNA was generated from total RNA. Quantitative real-time PCR was performed according to the supplier protocols (Perkin-Elmer). Oligonucleotides used as forward primer (F), reverse primer (R), and probes (P) labeled with the fluorescent quencher TAMRA (3′) and the indicator dye FAM (5′) were as follows: for mouse intracellular adhesion molecule (ICAM)-1, F: 5′-GGGACACGGAGGCACTAT-3′; R: 5′-GCTTGTGAGTGTTGACTCAGA-3′; P: 5′-FAM-CAGG-CCGCAAGGGCTGTC-TAMRA-3′; for mouse monocyte-chemoattractant protein (MCP)-1, F: 5′-TCTGGGCTCTGCTGTTACA-3′; R: 5′-CCAGCTACTACCTGAGATCC-3′; P: 5′-FAM-CCACGCACCTGCTGCTACACCA-TAMRA-3′; and for mouse Vascular cellular adhesion molecule (VCAM)-1, F: 5′-CCTTTGCTGAATGCAAGGA-3′; R: 5′-TGGGACCTCCAGTACCTC-3′; P: 5′-FAM-AAACGAAAAGTTCTGCTGACAA GTCCCT-TAMRA-3′. The copy numbers were calculated from plasmid cDNA standards containing the PCR amplicon. ICAM-1, MCP-1, and VCAM-1 levels were expressed as copy number per 1000 copies of hypoxanthine transferase amplified as described elsewhere.7

Statistical Analysis

The significance of differences between C57BL6, LDLR−/−, ob/ob, double-mutant mice was determined in Kruskal-Wallis test (Graph Pad Prism version 3.02) followed by Dunn’s Multiple Comparisons test. P<0.05 was considered statistically significant.
**Results**

**Blood Analysis**

Compared with C57BL6 mice, LDLR<sup>−/−</sup> and double-mutant mice had higher non-HDL and HDL cholesterol levels (P<0.05 and <0.001, respectively) (Table). Double-mutant mice had higher triglyceride levels than the other strains. In agreement with earlier findings, non-HDL cholesterol consisted of VLDL, IDL, and LDL in double-mutant mice and primarily of LDL in LDLR<sup>−/−</sup> mice. Compared with C57BL6 mice, ob/ob and double-mutant mice had higher HDL cholesterol levels (P<0.05 and <0.001, respectively). HDL cholesterol was higher in double-mutant than in ob/ob and LDLR<sup>−/−</sup> mice (P<0.05 and <0.001, respectively). The titer of autoantibodies against MDA-modified LDL was significantly higher in double-mutant mice than in C57BL6, ob/ob, and LDLR<sup>−/−</sup> mice (P<0.001). Fasting and nonfasting blood glucose levels in C57BL6 and LDLR<sup>−/−</sup> mice were significantly lower than in ob/ob and LDLR<sup>−/−</sup>;ob/ob mice (Table).

**Atherosclerosis and Macrophage Homing**

Figure 1 shows representative Sudan IV–stained en face preparations of thoracic abdominal aortas (A through C) and oil-red O–stained cross-sections of the aortic root (D through F) of 22- to 26-week-old LDLR<sup>−/−</sup>, ob/ob, and double-mutant mice. Figures 1G through 1I show sections of double-mutant mice immunostained for macrophages, oxidized LDL, and myeloperoxidase (I). Line=1 cm.

<table>
<thead>
<tr>
<th></th>
<th>C57BL6</th>
<th>LDLR&lt;sup&gt;−/−&lt;/sup&gt;</th>
<th>Ob/ob</th>
<th>LDLR&lt;sup&gt;−/−&lt;/sup&gt;;ob/ob</th>
<th>ANOVA</th>
<th>P</th>
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<tr>
<td>Total cholesterol, mg/dL</td>
<td>78 ± 19</td>
<td>213 ± 68</td>
<td>136 ± 51</td>
<td>971 ± 201</td>
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<td>&lt;0.001</td>
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<td>Non-HDL cholesterol, mg/dL</td>
<td>37 ± 18</td>
<td>156 ± 52</td>
<td>69 ± 36</td>
<td>878 ± 202</td>
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<td>&lt;0.001</td>
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<tr>
<td>HDL cholesterol, mg/dL</td>
<td>41 ± 20</td>
<td>57 ± 35</td>
<td>67 ± 18</td>
<td>93 ± 12</td>
<td></td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Triglycerides, mg/dL</td>
<td>23 ± 5</td>
<td>65 ± 20</td>
<td>29 ± 7</td>
<td>536 ± 280</td>
<td></td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Titer of autoantibodies against MDA-modified LDL</td>
<td>1.04 ± 0.13</td>
<td>1.49 ± 0.27</td>
<td>1.90 ± 0.64</td>
<td>4.03 ± 1.23</td>
<td>&lt;0.001</td>
<td></td>
</tr>
<tr>
<td>Fasting glucose, mg/dL</td>
<td>77 ± 15</td>
<td>83 ± 8.9</td>
<td>183 ± 51</td>
<td>182 ± 36</td>
<td></td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Fasting insulin, μg/L</td>
<td>0.44 ± 0.35</td>
<td>0.37 ± 0.27</td>
<td>11 ± 2</td>
<td>19 ± 10</td>
<td></td>
<td>&lt;0.001</td>
</tr>
</tbody>
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Data are mean±SD.
myeloperoxidase. Macrophage-rich areas are costained with apoB-containing lipoproteins and myeloperoxidase (Figures 1G though 1I).

Age-matched C57BL6 and ob/ob mice had no detectable lesions. Lesions covered 20±1.7% of total area of the thoracic abdominal aorta from double-mutant mice compared with 3.5±1.1% in LDLR<sup>−/−</sup> (Figure 2A). Plaque volumes in the aortic arch of double-mutant mice were 10 times larger than in LDLR<sup>−/−</sup> and ob/ob mice (Figure 2B). Plaque areas immunostained for oxidized LDL were 7.7 times larger, whereas those immunostained for macrophages were 13 times larger (P<0.001 for both). More macrophages were found in the aortic root of 22- to 26-week-old double-mutant mice than in C57BL6, LDLR<sup>−/−</sup>, and ob/ob mice (Figure 2C). Correlation between number of homed macrophages and mean lesion area was 0.85 (Spearman r; P<0.0001).

### Figure 2

Lesion area in the thoracic abdominal aorta (A) and plaque area in ~12 sections of the aortic arch (B) was measured by computer-assisted image analysis. Plaque volumes were calculated as mean plaque area multiplied by plaque length. Data are mean±SEM for 14 to 17 mice per group. C, Four days after intravenous injection, fluorescein-labeled macrophages in the aortic root were counted. **P<0.01; ***P<0.001 versus C57BL6. Data are mean±SEM for 9 to 10 mice per group.

### Figure 3

VCAM-1 (A), ICAM-1 (B), and MCP-1 (C) mRNA expression in the aorta from 12- to 14-week-old LDLR<sup>−/−</sup>, ob/ob, and LDLR<sup>−/−</sup>;ob/ob was measured by quantitative RT-PCR. Data are mean±SEM for 10 to 12 mice per group.

### Adhesion Molecule Expression in Mouse Aorta

Expression of VCAM-1 and ICAM-1 mRNA in the aorta of 12- to 14-week-old LDLR<sup>−/−</sup>;ob/ob mice was increased compared with LDLR<sup>−/−</sup> and ob/ob mice. At this age, we did not detect atherosclerotic lesions in any of the mouse strains. There was a trend for increased MCP-1 mRNA expression; however, this did not reach statistical significance (Figure 3).

### In Vitro Cellular Cholesterol Efflux

The total in vitro cellular cholesterol efflux induced by HDL of the different strains of mice was similar. The fractional rate of cholesterol efflux was also not different (Figure 4).

### PAF-AH, PON Activity, and LCAT Activity

HDL-associated PAF-AH activity, adjusted for total HDL phospholipid, was similar among the 4 groups of mice (Figure 5A). PON activity, adjusted for total HDL phospholipid, was significantly lower in double-mutant mice compared with LDLR<sup>−/−</sup> (P<0.01), ob/ob, and C57BL6 mice (P<0.001 for both) (Figure 5B). HDL-associated LCAT activity, adjusted for total HDL-phospholipid, in double-
mutant mice was lower than in C57BL6 and LDLR−/− mice (P<0.001 for both) (Figure 5C). There was a trend for decreased LCAT activity compared with ob/ob mice, but it did not reach statistical significance.

**LCAT Gene Transfer**

At day 0, LCAT activity was 1.5±0.1% CE/0.5 hours. AdRR5 had no effect on plasma LCAT activity. LCAT gene transfer resulted in a 64% increase in LCAT activity at day 7 (P=0.0025) (Figure 6A). This increase did not result in a change of HDL cholesterol levels (96±20 mg/dL in LCAT-treated versus 87±21 mg/dL in RR5-treated mice) or in a change of the cholesterol ester to free cholesterol ratio (2.77±0.72 versus 2.61±0.64). However, it was associated with a 40% reduction in titer of autoantibodies against MDA-modified LDL (Figure 6B). Six weeks after adenovirus injection in female LDLR−/−;ob/ob mice, a 40% reduction of the oxidized LDL area, a 50% reduction of macrophage area, a 44% reduction in plaque area in the thoracic abdominal aorta (Figure 6C), and a 42% reduction in plaque volume in the aortic root (Figure 6D) was observed in AdLCAT-treated mice compared with AdRR5-treated mice.

**Discussion**

Increased oxidation of atherogenic apoB-containing lipoproteins in the circulation and the arterial wall and impaired HDL-associated antioxidant defense in obese dyslipidemic mice were associated with increased expression of monocyte-specific adhesion molecules in the aorta, macrophage homing, and accelerated atherosclerosis. Adenovirus-mediated LCAT gene transfer reduced oxidation of atherogenic apoB-containing lipoproteins in the circulation and the arterial wall. This was associated with decreased accumulation of macrophages in the aortic root and thereby atherosclerosis.

**Model Choice**

Our findings are in agreement with those of Hasty et al that severe LDL-hypercholesterolemia, hypertriglyceridemia, and insulin resistance characterize LDLR−/−;ob/ob mice. The latter is also seen in hyperleptinemic models of obesity, such as the agouti mouse or the brown adipose tissue–deficient mouse, and in diet-induced obesity. However, none of these animals have dyslipidemia. Thus, the LDLR−/−;ob/ob mouse is a unique model to study mechanisms underlying higher susceptibility to atherosclerosis in association with dyslipidemia and obesity.

**Oxidation of LDL and Macrophage Accumulation**

Oxidized LDL has been implicated to be involved in early atherogenesis. Among others have demonstrated an association between the oxidation of LDL and coronary artery disease. We have shown that dyslipidemia in association with obesity is an independent predictor of
circulating oxidized LDL in persons without clinical evidence of cardiovascular disease. Because the assay for oxidized LDL in blood is based on a mouse monoclonal antibody, oxidized LDL cannot be measured directly in mouse blood. Previously, the titer of autoantibodies against MDA-modified LDL has been used as a proxy for oxidized LDL in mice. Compared with ob/ob and LDLR−/− mice, the double-mutant mice had higher titers of autoantibodies. We also demonstrated increased oxidation of apoB-containing lipoproteins in the aortic root of double-mutant mice. We observed increased expression of ICAM-1 and VCAM-1 mRNA in the aorta of 12- to 14-week-old double-mutant mice. This is before atherosclerotic lesions were detectable, suggesting that monocyte infiltration is an early event in atherosclerosis in double-mutant mice. This increased expression of adhesion molecules can explain the increased in vivo macrophage homing resulting in increased accumulation of macrophage foam cells in double-mutant mice. Thus, deposition of oxidized lipoproteins and infiltration of macrophages in the arterial wall are key events in the development of atherosclerosis in LDLR−/−:ob/ob mice. Most likely, oxidized LDL does not originate from extensive metal ion–induced oxidation in the blood but from mild oxidation by cell-associated enzymes such as myeloperoxidase that colocalized with macrophages and oxidized apoB-containing lipoproteins in the aortic root of double-mutant mice.

HDL and Prevention of LDL Oxidation
HDL-associated PAF-AH activity was not statistically different among the 4 groups of mice, although it was higher in total plasma of double-mutant mice. However, the total plasma PAF-AH activity was higher because of a much higher level of PAF-AH in association with apoB-containing lipoproteins (data not shown). Thus, it may be envisioned that PAF-acetylhydrolase associated with apoB-containing lipoproteins, in contrast to HDL-associated PAF-acetylhydrolase, is not protective against atherosclerosis. It has also been shown that plasma PAF-AH activity does not reflect the activity of PAF or PAF-like lipids in the plaque.

Serum and HDL-associated paraoxonase activity was lower in double-mutant mice, suggesting impaired protection against lipoprotein oxidation, resulting in the formation of lipid peroxides, cholesterol linoleate hydroperoxides, and hydrogen peroxide that inhibits paraoxonase.

LCAT, another HDL-associated enzyme, also prevents the oxidation of LDL, and oxidized LDL inhibits LCAT activity. Double-mutant mice showed lower plasma LCAT activity. Adenovirus-mediated overexpression of human LCAT in double-mutant mice decreased oxidation of apoB-containing lipoproteins in the circulation, evidenced by the decrease in titer of autoantibodies against MDA-modified LDL, and reduced accumulation of oxidized lipoproteins and of macrophages in the aortic arch and protected them from atherosclerosis. Thus, our findings are in agreement with data showing a direct relation between LCAT deficiency and risk for atherosclerosis.

The higher HDL cholesterol levels in double-mutant mice did not result in an increased cellular cholesterol efflux from macrophages, the first step in the reverse cholesterol transport. We cannot exclude that the subsequent step in this process, where LCAT is involved, was inhibited in the double-mutant mice. However, we do know that increased LCAT activity was not associated with an increase in HDL cholesterol or increased cholesterol esterification.

Limitations of the Study
We have studied atherosclerosis in mice, and mechanisms of murine atherosclerosis can be different from these of human atherosclerosis. Furthermore, double-mutant mice had very high cholesterol levels, whereas most obese persons do not. However, the increased oxidative stress in obese dyslipidemic mice is in agreement with the increased oxidation of LDL in obese people.

Conclusion
Obese dyslipidemic mice are characterized by increased oxidation of apoB-containing lipoproteins in the blood and the aortic root and impaired HDL-associated antioxidative
defense. LCAT gene transfer can partially prevent atherosclerosis in the absence of a change in HDL cholesterol level. The lower titer of autoantibodies against oxidatively modified LDL and the reduced accumulation of oxidized lipoproteins in the aorta of LCAT-treated mice suggest that the protective effect of LCAT is partly attributable to its antioxidant action.

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References

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