Amino Terminal 38.9% of Apolipoprotein B-100 Is Sufficient to Support Cholesterol-Rich Lipoprotein Production and Atherosclerosis

Zhouji Chen, Robin L. Fitzgerald, Jeffrey E. Saffitz, Clay F. Semenkovich, Gustav Schonfeld

Objective—Carboxyl terminal truncation of apolipoprotein (apo)B-100 and apoB-48 impairs their capacity for triglyceride transport, but the ability of the resultant truncated apoB to transport cholesterol and to support atherosclerosis has not been adequately studied. The atherogenicity of apoB-38.9 was determined in this study by using our apoB-38.9—only (Apob38.9/38.9) mice.

Methods and Results—ApoB-38.9–lipoproteins (Lp-B38.9) circulate at very low levels in Apob38.9/38.9 mice as small LDLs or HDLs. Disruption of apoE gene in these mice caused accumulation of large amounts of βVLDL-like Lp-B38.9 in plasma. These βVLDL particles were more enriched with cholesteryl esters but poor in triglycerides compared with the apoB-48-βVLDL of the apoB-wild-type/apoE-null (Apob+/+Apoe−−) mice. Likewise, apoB-38.9-βVLDL secreted by cultured Apob38.9/38.9 mouse hepatocytes also had higher ratios of total cholesterol to triglycerides than apoB-48-βVLDL secreted by the apoB-48-only hepatocytes. Thus, despite its impaired triglyceride-transporting capacity, apoB-38.9 has a relatively intact capacity for cholesterol transport. Spontaneous aortic atherosclerotic lesions were examined in apoB-38.9—only/apoE-null (Apob38.9/38.9 /Apoe−−) mice at ages 9 and 13 months. Extensive lesions were found in the Apob38.9/38.9 /Apoe−− mice as well as in their Apob+/+38.9 /Apoe−− and Apob+/+ /Apoe−− littermates.

Conclusion—Deleting the C-terminal 20% from apoB-48 does not impair its ability to transport cholesterol and to support atherosclerosis, thus narrowing the “atherogenic region” of apoB. (Arterioscler Thromb Vasc Biol. 2003;23:668-674.)

Key Words: atherosclerosis ■ apolipoprotein B ■ animal model ■ VLDL secretion ■ cholesterol secretion ■ liver

A polipoprotein (apo)B1 is an indispensable structural protein component of the triglyceride-rich VLDLs secreted by the liver and the chylomicrosomes secreted by the intestine.1,2 It plays a central role in lipid transport throughout the body. In addition to its normal lipid-transport functions, apoB is the major atherogenic apolipoprotein in humans and experimental animals.3–5 High levels of plasma apoB-containing lipoproteins are a well-established causal factor for the development of atherosclerotic diseases.3–5 Therefore, it is important to understand fully the structure–function relationship of apoB.

The full-length apoB (apoB-100) contains 4536 amino acid residues1,2 that are predicted to form a pentapartite structure composed of 3 amphipathic α-helical domains alternating with 2 β-strand domains.6,7 Because of a post-transcriptional modification of the apoB mRNA that converts codon 2153, CAA, to a stop codon, UAA, the apoB protein also exists in a truncated form containing the NH2 terminal 48% of apoB-100, designated apoB-48.8,9 In humans, apoB-48 is produced only by the intestine,1,10 whereas both the intestine and the liver in rodents secrete apoB-48.11,12 Thus, although apoB-100 is the predominant apoB isoform in human plasma,1,2 apoB-100 and apoB-48 circulate in mice at similar levels.11,12

In addition to the normal apoB isoforms, numerous forms of COOH terminally truncated apoB, with sizes ranging from apoB-27.6 to apoB-89, have been identified in the plasma of humans with familial hypobetalipoproteinemia (FHBL).2,13,14 They are the products of nonsense and frameshift mutations in the apoB gene (ApoB) that result in the formation of premature termination codons.2,13,14 These naturally occurring apoB genetic variants have provided valuable information on the structure–function relationship of apoB. For example, we demonstrated in vivo that COOH terminal truncation of apoB-100 and apoB-48 impairs their capability for triglyceride transport,15–18 resulting in accumulation of triglycerides in the liver of FHBL humans16,19–21 and mice.17,18 We and other investigators have also shown that the truncated apoB-containing lipoproteins are cleared rapidly from the plasma because of their enhanced interactions with the LDL receptor–mediated pathway22–24 and/or with an LDL receptor–independent megalin-mediated pathway.25,26 In contrast, little is known about the correlation between the size of apoB-38.9 and the plasma concentration.
an apoB variant and its ability to support atherogenesis. Nevertheless, the critical structural domains of apoB that are essential for the atherogenicity of apoB-containing lipoproteins may be located within the NH₂-terminal 48% of the apoB-100 because apoB-48 and apoB-100 appear to be equally atherogenic. Thus, it is particularly interesting and important to understand the function of those truncated apoB variants that are smaller than apoB-48 in cholesterol transport and atherogenesis. Such studies may lead to elucidation of the relationship between the apoB structure and the atherogenicity of apoB-containing lipoproteins. Unfortunately, FHBL subjects are not suitable models to address this issue because subjects homozygous for an apoB truncation mutation are very rare.

Recently, we created a mouse bearing a knocked-in single base pair deletion on its Apob at the position identical to that of a naturally occurring apoB-38.9 mutation in an FHBL kindred. This mouse may be a valuable animal model to define the atherogenicity of apoB-38.9-containing lipoproteins. Our previous studies demonstrated that apoB-38.9 has an impaired capacity for hepatic triglyceride transport, but its ability to form cholesterol-rich atherogenic lipoprotein particles is unknown. Thus, in the present study, we used this mouse to address the following two questions: Does apoB-38.9 also have an impaired cholesterol-transporting capacity? Do the apoB-38.9-containing lipoproteins promote atherosclerosis? To examine the capacity of apoB-38.9 for cholesterol transport, we assessed whether cholesterol-rich VLDL-sized apoB-38.9-containing lipoproteins accumulate in the plasma of apoB-38.9–only/apoE-null (Apob<sup>38.9/38.9</sup> Apoe<sup>−/−</sup>) mice. We also determined the lipid composition of the newly secreted apoB-38.9–VLDL. To assess the atherogenic potential of apoB-38.9–containing lipoproteins, we quantified spontaneous aortic atherosclerotic lesions in Apob<sup>38.9/38.9</sup> Apoe<sup>−/−</sup> mice. These studies establish the ability of apoB-38.9 to form cholesterol-rich lipoproteins and support atherosclerosis.

**Methods**

**Mice**

The apoB-38.9 mice have been described previously. They were produced with mouse embryonic stem cell technology by using conventional homologous recombination and Cre-loxp system. They have a mixed genetic background with 50% C57BL/6 and 50% 129/SvJ. ApoE-null mice were from The Jackson Laboratory (Bar Harbor, ME) and had a C57BL/6 background. Thus, offspring from crossbreeding of the apoB-38.9 mice with these apoE-null mice had a mixed genetic background with 75% C57BL/6 and 25% 129/SvJ. In some experiments, we also used apoB-48–only mice (The Jackson Laboratory) to isolate hepatocytes. They have a mixed genetic background with 50% C57BL/6 and 50% 129/SvJ.

All mice were weaned at 3 weeks of age, housed in a specific pathogen–free barrier facility with a 12-hour light/dark cycle, and fed a regular mouse chow diet (Ralston Purina, St. Louis, MO).

**Western Blot Detection of ApoB and Lipoprotein Fractionation**

To perform Western blot analysis, aliquots of mouse plasma were subjected to electrophoresis on 3% to 12% gradient SDS-PAGE gels under reducing conditions and electrottransferred onto Immobilon-P (Millipore Corp.). Western blot analyses were conducted by using rabbit antismouse apoB antiserum (1:10,000) or antilicultathione-s-transferase mouse apoB amino acids 26 to 289 and an ECL Western blot detection kit (Amersham Pharmacia Biotech, Inc.). The ECL signals were quantified by analyzing the density of the protein bands on x-ray film by using a Sigma gel computer software (SPPS Science Corp.).

A fast-performance liquid chromatography (FPLC) Superoxide column was used to assess the distribution of lipids and apoB within the lipoprotein fractions of mouse plasma as described previously. The distribution of apoB in each lipoprotein fraction was determined by Western blot analysis.

**Electron Microscopy of Plasma VLDL**

Plasma was collected from 20-week-old Apob<sup>38.9/38.9</sup> Apoe<sup>−/−</sup> and Apob<sup>−/−</sup>/Apoe<sup>−/−</sup> mice after fasting for 4 hours. VLDL (d<1.009) was isolated by ultracentrifugation. Negatively stained preparations of VLDL particles were performed as described previously. The stained VLDL particles were examined by using a transmission electron microscope.

**Analysis of ApoB-38.9– and ApoB-48–VLDL Secreted by Cultured Hepatocytes**

Hepatocytes were isolated from 8- to 10-week-old apoE wild-type apoB-38.9–only or apoB-48–only mice as described previously. Viability of the cells (≈80%) was determined by Trypan exclusion.

Cells were plated onto 150-mm dishes (1.5×10⁵ cells/dish) coated with poly-γ-lysine (Sigma) and incubated at 37°C under 5% CO₂ in 10% fetal bovine serum/DMEM. After 1 to 2 hours of attachment, the cell monolayers were washed twice and incubated in 10% fetal bovine serum/DMEM for 6 hours. Thereafter, cells were washed 3 times with serum-free DMEM. After washing, cells were incubated in 10 mL of DMEM containing 0.5 mmol/L oleic acid complexed with BSA (ratio of oleic acid to BSA = 3.6:1). Conditioned media were collected after a 6-hour incubation. Media from 4 dishes (ie, a total of 40 mL) from hepatocytes of Apob<sup>38.9/38.9</sup> or Apob<sup>48/48</sup> mice were pooled and adjusted to a density of d = 1.009 g/mL with KBr and VLDL was isolated by ultracentrifugation. The VLDL fractions were collected and lipids were extracted from the VLDL as described previously. Glycerol tri[1,4-C] oleate (59.0 Ci/mmol; Amersham Pharmacia Biotech, Inc.) and [1α, 2α (n)-H] cholesterol (39.0 Ci/mmol; Amersham Pharmacia Biotech, Inc.) were used as tracers for determination of recovery of lipids. The dried lipid extracts were dissolved in 1% Triton X-100 in chloroform, dried under a stream of N₂, and then the dried lipid-Triton X-100 complexes were solubilized in H₂O as described previously for determination of triglycerides, cholesterol, and phospholipids by using enzymatic kits (WAKO Chemicals USA, Inc.).
TABLE 1. Production of Viable Offspring

<table>
<thead>
<tr>
<th>Breeding Pair</th>
<th>Apob&lt;sup&gt;38.9&lt;/sup&gt;/Apoe&lt;sup&gt;-/-&lt;/sup&gt; × Apob&lt;sup&gt;38.9&lt;/sup&gt;/Apoe&lt;sup&gt;-/-&lt;/sup&gt;</th>
<th>Apob&lt;sup&gt;38.9&lt;/sup&gt;/Apoe&lt;sup&gt;-/-&lt;/sup&gt;</th>
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<td>56 / 78 / 5</td>
<td>670 Arterioscler Thromb Vasc Biol. April 2003</td>
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The values presented in this table were based on the data obtained from genotyping of the offspring 2 to 3 weeks after birth. The offspring from Apob<sup>38.9</sup>/Apoe<sup>-/-</sup> × Apob<sup>38.9</sup>/Apoe<sup>-/-</sup> had a mixed genetic background with 75% C57BL/6 and 25% 129/SvJ.

Quantification of Aortic Lesion Extent

An en face atherosclerotic lesion measurement technique was used to quantify the lesion extent on unstained, pinned-out aortas.33 Briefly, after exsanguination, the heart and aorta were perfused with PBS and fixed by perfusion with 4% paraformaldehyde. Thereafter, the aorta was incised longitudinally, dissected free from adventitia, and pinned flat. Images of the pinned aortas were captured by using a Nikon digital camera through a Nikon stereomicroscope and analyzed by using an image processing program.33 Lesion areas were estimated as percentage of involvement of the intimal surface area for the arch (extending from the aortic valve to a point just distal to the left subclavian artery), the thoracic aorta (extending to the last intercostal artery), and the abdominal aorta (extending to the iliac bifurcation).

Miscellaneous Procedures

Cellular protein contents were determined by using a modified Lowry method.35 Plasma lipids were determined by using enzymatic kits (WAKO Chemicals USA, Inc.).

Results

Production of ApoB-38.9–Only/apoE-Null Mice

ApoB-38.9 is present at a much lower level than apoB-48 in the apoB-38.9–heterozygous mice because of its faster clearance rate.17,18 However, we have previously18 shown that deletion of the Apoe in apoB-38.9–heterozygous (ApoB<sup>38.9</sup>/Apoe<sup>-/-</sup>) mice retarded catabolism of the apoB-38.9–containing lipoproteins. Thus, we anticipated that disruption of Apoe in apoB-38.9–only (ApoB<sup>38.9</sup>/Apoe<sup>-/-</sup>) mice would cause accumulation of apoB-38.9–containing lipoproteins in plasma and would allow us to examine their physicochemical characteristics and atherogenicity in vivo. The Apob<sup>38.9</sup>/Apoe<sup>-/-</sup> mice were intercrossed to generate Apob<sup>38.9</sup>/Apoe<sup>-/-</sup> mice. However, of 139 offspring, there were only 5 Apob<sup>38.9</sup>/Apoe<sup>-/-</sup> mice (Table 1), amounting to less than 10% of the number of apoB–wild-type/apoE-null (ApoB<sup>-/-</sup>/Apoe<sup>-/-</sup>) littermates. This is in sharp contrast to survival rate of Apob<sup>38.9</sup>/Apoe<sup>-/-</sup> mice on a normal mouse chow diet. Plasma cholesterol levels of the Apob<sup>38.9</sup>/Apoe<sup>-/-</sup> mice were similar to those of the Apob<sup>-/-</sup>/Apoe<sup>-/-</sup> and Apob<sup>38.9</sup>/Apoe<sup>-/-</sup> littermates. Compared with Apob<sup>38.9</sup>/Apoe<sup>-/-</sup> mice, plasma apoB-38.9 levels in Apob<sup>38.9</sup>/Apoe<sup>-/-</sup> mice increased 10-fold (Figure 1).

The Apob<sup>38.9</sup>/Apoe<sup>-/-</sup> mouse plasma was subjected to FPLC analysis to determine the distribution of cholesterol in each lipoprotein fraction. Like the Apob<sup>-/-</sup>/Apoe<sup>-/-</sup> mouse plasma cholesterol, the majority of the cholesterol from Apob<sup>38.9</sup>/Apoe<sup>-/-</sup> mouse plasma was eluted at the VLDL peak (fractions 5 to 14) (Figure 2). The distribution of apoB-38.9 protein in the lipoprotein fractions of the Apob<sup>38.9</sup>/Apoe<sup>-/-</sup> mouse was also in a pattern similar to those of apoB-48 in Apob<sup>-/-</sup>/Apoe<sup>-/-</sup> or Apob<sup>38.9</sup>/Apoe<sup>-/-</sup> mice (Figure 2). This is in sharp contrast to the distribution of apoB-38.9 in plasma of Apob<sup>38.9</sup>/Apoe<sup>-/-</sup> and Apob<sup>38.9</sup>/Apoe<sup>-/-</sup> mice, in which apoB-38.9 was eluted only as LDL–HDL–sized particles (fractions 22 to 32; Figure 2, panel D and the references 17,18). On negative-staining electron microscopy, the apoB-38.9–βVLDL particles from the Apob<sup>38.9</sup>/Apoe<sup>-/-</sup> mice appeared very heterogeneous in size, with particle diameters ranging from 10 to 200 nm. The median diameter of these apoB-38.9–βVLDL particles was approximately 45 nm, which is similar in size to apoB-48–βVLDL particles. The negatively stained apoB-38.9–βVLDL and apoB-48–βVLDL particles appeared to be spherical and similar in shape. Chemical analysis of apoB-38.9–βVLDL particles revealed that they were relatively more enriched with cholesteryl esters but triglyceride-poor than the apoB-48–βVLDL particles (ratios of cholesteryl ester to triglyceride: 23 for apoB-38.9–βVLDL versus 15 for apoB-48–βVLDL).

Mouse plasma has very little cholesteryl ester–transfer protein activity,36 which is responsible for transferring cholesteryl esters from HDLs to LDLs in humans.37 Thus, the presence of the cholesterol-rich apoB-38.9–βVLDL in Apob<sup>38.9</sup>/Apoe<sup>-/-</sup> mouse plasma suggests that apoB-38.9 may be capable of assembling cholesterol-rich VLDLs in the...
liver and intestine. To examine this possibility, VLDLs (d<1.009 g/mL) were isolated from conditioned media from cultured apoB-48–only or apoB-38.9–only hepatocytes and analyzed for lipid composition. As shown in Table 2, the apoB-38.9–VLDL was relatively triglyceride poor but cholesterol rich compared with apoB-48–VLDL.

Together, these results demonstrate that despite its impaired capability to transport triglycerides, apoB-38.9 has a relatively intact ability to transport cholesterol. This finding suggests that distinct domains on the apoB protein may be responsible for recruiting triglycerides and cholesterol into apoB-lipoproteins during VLDL assembly.

Atherogenic Potential of ApoB-38.9–Containing Lipoproteins

To determine whether apoB-38.9 is capable of supporting atherogenesis, spontaneous aortic lesion extent was measured in the 4 Apob38.9/38.9/Apoe−/− mice, 2 at age 9 months and 2 at 13 months. Apob+/+/Apoe−/− and Apob38.9/38.9/Apoe−/− littermates with plasma cholesterol levels similar to those of Apob38.9/38.9/Apoe−/− mice were used as controls. At both ages, extensive lesions were seen in the aortas of Apob38.9/38.9/Apoe−/− mice as well as their littermate controls (Figure 3). At 13 months of age, Apob38.9/38.9/Apoe−/− mice tended to have larger lesions than the control mice (Figure 3, panels B and C). Plasma levels of apoA-I and apoA-IV were not affected by the apoB-38.9 mutation in the apoE-null mice (Figure 4), indicating that the atherosclerotic development in the Apob38.9/38.9/Apoe−/− mice was caused by accumulation of atherogenic lipoproteins rather than by reduced levels of antiatherogenic agents, such as apoA-I35,39 and apoA-IV.40,41 Collectively, these results established direct evidence demonstrating the atherogenicity of apoB-38.9–containing lipoproteins.

Discussion

ApoB is a major atherogenic apolipoprotein in humans and experimental animal models.1–5 The atherogenicity of apoB may be dependent on the ability of apoB to carry cholesterol and on its susceptibility to retention in the subendothelial space and the subsequent oxidative modification and uptake by macrophages in the vessel wall.4,42–44 Current understanding of the structure–function relationship of apoB with respect to these atherogenic processes is still incomplete. It has been shown that apoB-48–containing lipoproteins are as atherogenic as the apoB-100–containing lipoproteins,27 indicating that the “atherogenic domains” of apoB-100 may be located within the N-terminal 48% of apoB-100. We now provide in vivo evidence that apoB-38.9, the N-terminal 38.9% of apoB-100, is fully atherogenic, thus narrowing the “atherogenic region” of apoB.

By using our previously generated apoB-38.9 mice bred onto an apoE-null background, we for the first time demonstrated that apoB-38.9 is capable of forming cholesterol-rich βVLDL-like lipoprotein particles in mouse plasma. We also showed that apoB-38.9–VLDL secreted by cultured Apob38.9/38.9 hepatoocytes was relatively triglyceride poor but cholesterol rich compared with the apoB-48–VLDL secreted by the Apob38.9/38.9 hepatoocytes. We have previously shown that apoB-38.9 has a much smaller capacity for triglyceride transport than apoB-48.17 The apoB-38.9–specifying mutation caused accumulation of triglycerides but not cholesterol or cholesteryl esters in the liver of the mutant mice,17 further suggesting that apoB-38.9 may have a normal capacity for hepatic cholesterol export via VLDL secretion. Thus, C-terminal truncation of apoB appears to exert differential effects on its ability to transport triglycerides and cholesterol, implying that distinct peptide regions within apoB-48 may be responsible for transporting different lipid classes. In contrast with apoB-38.9, apoB-27.6 is incapable of assembling buoyant lipoprotein particles.18 Furthermore, disruption of apoE gene did not cause accumu-
lation of plasma of apoB-27.6–lipoproteins in the apoB-27.6 mice,18 nor did it alter the density distribution of apoB-27.6–lipoproteins in the plasma (data not shown). Taken together, these observations suggest that the peptide region between COOH termini of apoB-27.6 and apoB-38.9 may have specific roles in cholesterol transport whereas the region between the COOH-termini of apoB-38.9 and apoB-48 may be more important for triglyceride transport. Compared with the nascent apoB-48– or apoB-38.9–containing VLDL particles found in the media of cultured hepatocytes, plasma apoB-48– or apoB-38.9–containing βVLDL particles circulating in the apoE-deficient mice contain much more cholesteryl esters and less unesterified cholesterol (data not shown). HDL is not a source of cholesterol esters for plasma apoB-containing lipoproteins in mouse because mouse plasma does not have cholesteryl ester transfer protein.36 However, a recent study has shown that lecithin-cholesterol acyltransferase catalyzed esterification of cholesterol found in the nascent apoB-38.9–VLDL particles.

The assembly of VLDL and chylomicrons is a complex process that brings together the amphipathic apoB polypeptide and 4 different classes of lipids, including surface lipid components (phospholipids and unesterified cholesterol) and a neutral lipid core composed of triglycerides and cholesterol esters. It is not known how the apoB-27.6–apoB-38.9 and the apoB-38.9–apoB-48 peptide segments exert their specific roles in cholesterol and triglyceride recruitment. It has been suggested that VLDL assembly undergoes a 2-step process,10,46,47 with a majority of triglycerides being recruited in the second step.46,47 It is possible that the apoB-38.9–apoB-48 peptide segment is particularly important for recruiting triglycerides during the second step of VLDL biogenesis whereas apoB-27.6–apoB-38.9 segment is sufficient for cholesterol/cholesteryl ester recruitment. A previous study by McLeod et al48 demonstrated that several β-sheet regions within the human apoB-27–apoB-48 were capable of assembling VLDL particles when they were fused to apoAI at the C-terminus, indicating that these apoB peptide segments are important for the lipid recruitment during the second step of
Apob mice were responsible. Because of the limited number of Apob/H11001–Apoe binding function of apoB may have an important role in lipoproteins, but it is generally thought that the proteoglycan-apoB protein is essential for the atherogenicity of apoB-lipoproteins. In fact, it was recently shown that the principal proteoglycan-binding site of human apoB-48–lipoproteins may be located within the first 100 residues of apoB. This apoB peptide segment is also involved in interactions of human apoB-100–LDL with proteoglycans. The putative proteoglycan-binding motifs in this region are conserved between human and mouse apoB proteins (Z. Chen, unpublished data). Thus, the principal proteoglycan-binding site of mouse apoB-38.9– and apoB-48–containing lipoproteins may also be located within the NH2 terminal 100 residues. Another important aspect of the atherogenic process is the uptake of the native and/or oxidized apoB-lipoproteins by macrophages in the arterial wall. Recent studies suggest that the oxidized phospholipids of oxidized apoB-lipoproteins play important roles in mediating the uptake of these apoB-lipoproteins by macrophages via scavenger receptors. The role of apoB in this process is poorly understood. However, if any specific apoB structural domain(s) is required for this process, apoB-38.9 apparently contains such domain(s).

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