Increased Atherosclerosis in Mice Lacking Apolipoprotein A-I Attributable to Both Impaired Reverse Cholesterol Transport and Increased Inflammation

Ryan E. Moore, Mohamad Navab, John S. Millar, Francesca Zimetti, Susan Hama, George H. Rothblat, Daniel J. Rader

Abstract—To test the hypothesis that apolipoprotein A-I (apoA-I) functions specifically to inhibit atherosclerosis independent of the level of high-density lipoprotein cholesterol (HDL-C) by promoting both reverse cholesterol transport and HDL antiinflammatory function in vivo, we established a murine atherosclerosis model of apoA-I deficiency in which the level of HDL-C is well maintained. ApoA-I−/− mice were crossed with atherosclerosis-susceptible low-density lipoprotein receptor−/−/apolipoprotein E−/− (LA) mice to generate LA mice with apoA-I+/+, apoA-I−/+, and apoA-I−/− genotypes. There were no major differences in the amounts of non–HDL-C and HDL-C in the plasma between different apoA-I genotypes. A significant inverse relationship was observed, however, between apoA-I gene dose and atherosclerosis in both female and male mice. Compared with LA-apoA-I−/+ mice, serum from LA-apoA-I−/− mice had a significantly reduced capacity to function as an acceptor of ABCA1- and SR-BI-mediated cellular cholesterol efflux, and also had markedly reduced lecithin cholesterol acyltransferase activity. In addition, LA-apoA-I−/− mice had significantly reduced macrophage-derived cholesterol esterification and reverse cholesterol transport in vivo. There was significantly reduced plasma paraoxonase (PON-1) activity, impaired HDL vascular antiinflammatory function, and increased basal levels of monocyte chemotactic protein-1 in the plasma of LA-apoA-I−/− mice compared with LA-apoA-I+/+ mice. In LA-apoA-I−/− mice, there was also greater induction of some, but not all, inflammatory cytokines and chemokines in response to intraperitoneal injection of lipopolysaccharide than in LA-apoA-I−/− mice. We conclude that apoA-I inhibits atherosclerosis by promoting both macrophage reverse cholesterol transport and HDL antiinflammatory function, and that these antiatherogenic functions of apoA-I are largely independent of the plasma level of HDL-C in this mouse model. (Circ Res. 2005;97:763-771.)

Key Words: antioxidant enzymes ■ atherosclerosis ■ lipoproteins ■ apolipoprotein A-I

Plasma levels of high-density lipoprotein cholesterol (HDL-C) are inversely associated with coronary heart disease risk.1 HDL is believed to be anti-atherogenic; however, the mechanisms by which it functions to inhibit atherosclerosis are not fully understood.2 HDL is believed to participate in reverse cholesterol transport and may also mediate antiinflammatory and antioxidant effects.2 Apolipoprotein A-I (apoA-I) is the primary protein component of mature HDL, and overexpression of apoA-I in animals raises HDL-C levels and reduces atherosclerosis.2 The specific nature of the contribution of apoA-I to HDL anti-atherogenic function, however, remains uncertain. Our previous work demonstrated that chow-fed apoA-I deficient mice on a low-density lipoprotein receptor (LDLr)−/− background developed increased atherosclerosis despite well-maintained levels of HDL-C, suggesting that apoA-I−/− deficient HDL may have reduced anti-atherogenic properties.3 To test the hypothesis that apoA-I is crucial for the anti-atherogenic properties of HDL and to explore the mechanisms by which this is true, we established a murine model of apoA-I deficiency in which the levels of HDL-C did not differ greatly between different apoA-I genotypes. ApoA-I−/− mice were crossed with atherosclerosis-susceptible LDLr−/−/apolipoprotein E−/− (LA) mice to generate LA mice with apoA-I+/+, apoA-I−/+, and apoA-I−/− genotypes. In this model, we observed a significant inverse relationship between apoA-I gene dose and atherosclerosis, despite only modest differences in the levels of HDL-C between groups. We also observed that the absence of apoA-I impairs macrophage reverse cholesterol transport and HDL antiinflammatory and antioxidant function in vivo, even in the presence of preserved HDL-C levels.
**Materials and Methods**

**Animals**

LA and apoA-I \(^{−/−}\) mice, both on a C57BL/6 background, were crossed to generate F1 LDLR \(^{−/−}\)/apoBec \(^{+/−}\)/apoA-I \(^{−/−}\) mice. F1 mice were crossed and genotyped for LDLR, apobec, and apo-A-I using polymerase chain reaction assays. LDLR \(^{−/−}\)/apoBec \(^{+/−}\)/apoA-I \(^{−/−}\) mice were crossed and genotyped for LDLR, apobec, and apoA-I using polymerase chain reaction assays. LDLR \(^{−/−}\)/apoBec \(^{+/−}\)/apoA-I \(^{−/−}\) (LA-apoA-I \(^{−/−}\)), LDLR \(^{−/−}\)/apoBec \(^{+/−}\)/apoA-I \(^{−/−}\) (LA-apoA-I \(^{+/−}\)), and LDLR \(^{−/−}\)/apoBec \(^{+/−}\)/apoA-I \(^{−/−}\) (LA-apoA-I \(^{+/−}\)) mice were identified for use in the study. Male and female mice were fed a chow diet for the duration of study. Mice were anesthetized using isoflurane for nonterminal collection of blood via the retro-orbital plexus using heparinized capillary tubes. Plasma was immediately isolated from whole blood and stored at \(-80^\circ\)C before use. Mice were euthanized by intraperitoneal injection of ketamine/xylazine for collection of plasma and quantitation of atherosclerosis at 3 or 10 months age. Mice were handled according to the guidelines of the University of Pennsylvania Institutional Animal Care and Use Committee.

**Isolation of Lipoproteins and Lipoprotein Analysis**

Plasma total cholesterol (TC), HDL-C, triglyceride, and phospholipids levels were measured on a Cobas Fara (Roche Diagnostics System Inc) using commercially available reagents from Wako Pure Chemical Industries and Trinity Biotech. Non–HDL-C was calculated as the difference between TC and HDL-C.

Plasma was separated using FPLC gel filtration (Amersham Pharmacia Biotech) on 2 Superose 6 columns as described previously.1 Western Blotting of FPLC fractions for apoA-II was performed as described.14 The very low-density lipoprotein (VLDL), LDL, and HDL cholesterol FPLC peaks from LA-apoA-I \(^{−/−}\) mice were deconvoluted from the FPLC profile by fitting the sum of 3 Gaussian curves using commercially available software (SAAM II (SAAM Institute)). Lipoproteins were isolated from pooled plasma by ultracentrifugation (d <1.006 g/mL for VLDL, d =1.019 to 1.063 g/mL for LDL/LDL, and d =1.063 to 1.21 g/mL for HDL) for compositional analysis. Lipoprotein composition was determined using commercially available enzymatic kits from Wako Pure Chemical Industries (Cholesterol CII kit, Free Cholesterol kit, Phospholipids B kit, Triacylglycerol kit) and from Pierce Biotechnology (Micro BCA Protein Assay kit). Lipoproteins loaded by equal phospholipid mass were subjected to SDS-PAGE under reducing conditions and stained for protein with GelCode Blue Stain Reagent (Pierce) for determination of apolipoprotein composition.

**Western Blotting**

Western Blotting of FPLC fractions for apoA-II was performed as described previously.1 Western blotting for the determination of PON-1 mass in plasma was performed as described previously.3

**Atherosclerosis**

Determination of the extent of atherosclerosis in the aortic root and Oil Red O staining were performed as previously described.3 Trichrome staining was performed using the multi-step method as described in the manufacturer’s instructions (Sigma, HT15).

**Cellular Cholesterol Efflux**

ABCA1- and SR-BI–mediated cholesterol efflux capacity of whole serum was assessed by the addition of the test sera diluted to 2%, as previously described.6,7

**In Vivo Reverse Cholesterol Transport**

Studies were performed as described.8 Briefly, \(^{3}H\)-cholesterol–labeled J774 foam cells were injected intraperitoneally. After macrophage injection, blood was collected at 3, 6, 12, and 24 hours via the retro-orbital plexus and at 48 hours via the vena cava. Plasma was used for liquid scintillation counting. Feces were collected continuously from 0 to 48 hours and lipids were extracted as described previously.9 Extracts were used for liquid scintillation counting to determine total \(^{3}H\)-cholesterol in the feces. Alternatively, fecal samples were rehydrated by the addition of water and ethanol, homogenized, and used directly for liquid scintillation counting to determine total \(^{3}H\)-lipids in the feces. The distribution of cholesterol between free and esterified forms in plasma was measured by thin-layer chromatography as described.10

**Enzyme Activity Assays**

PON-1, platelet-activating factor acetylhydrolase (PAF-AH), and lecithin-cholesterol acyltransferase (LCAT) activity assays were performed on whole plasma or FPLC fractions as described previously.11,12 LCAT activity was measured using an exogenous proteoliposome substrate containing \(^{14}C\)cholesterol.13

**Human Artery Wall Cocultures and Monocyte Chemotaxis Assay**

Human monocytes were prepared and chemotaxis assays were performed as described.14 The cocultures were treated for 8 hours with native human LDL (100 \(\mu\)g of LDL cholesterol/mL) in the absence or presence of mouse HDL (50 \(\mu\)g of HDL-C/mL) that was isolated by FPLC (fractions 30 to 36 for LA-apoA-I \(^{−/−}\) and LA-apoA-I \(^{+/−}\) mice, fractions 27 to 33 for LA-apoA-I \(^{−/−}\) mice, Figure 1). Alternatively, mouse LDL isolated by FPLC (fractions 12 to 22 for all groups) was added to the coculture in the absence of any HDL for 8 hours. The supernatants were removed and the cocultures washed, and fresh culture medium 199 (M199; Gibco) was added and incubated for an additional 8 hours. At the end of incubation, the supernatants were collected from cocultures and assayed for monocyte chemotactic activity as described.14
Measurement of Cytokines and Chemokines
A multiplexed, sandwich-type ELISA assay was used for the measurement of cytokines and chemokines in plasma (Pierce Biotechnology) as described.15

Statistical Analysis
Values are presented as mean±SEM. Results were analyzed by ANOVA, Student’s t test, Mann-Whitney test, or Fisher’s exact test where appropriate with the use of GraphPad Prism Software.

Results
Plasma Lipids and Lipoproteins
At 10 months of age, TC, non–HDL-C, and phospholipid levels were similar in female mice from each apoA-I genotype group (Table 1 and Figure 1). In male (but not female) LA-apoA-I+/+ and LA-apoA-I−/− mice there was a modest increase in TC, non–HDL-C, and phospholipid levels compared with LA-apoA-I+/− mice (Table 1). Triglycerides were modestly increased in both female and male LA-apoA-I+/+ and LA-apoA-I−/− mice compared with LA-apoA-I+/− mice (Table 1). HDL-C levels determined by standard precipitation of apoB-containing lipoproteins were slightly reduced in female and male LA-apoA-I−/− mice (20% or 14% respectively) compared with LA-apoA-I+/− mice (Table 1). HDL-C levels in LA-apoA-I−/− mice were intermediate between the levels present in LA-apoA-I+/+ and LA-apoA-I−/− mice. It is notable that on the LA background, HDL-C levels are well maintained in LA-apoA-I−/− mice (86 to 95 mg/dL), higher than those in wild-type mice, and far exceeding HDL-C levels in apoA-I−/− mice on a wild-type background (∼15 mg/dL).3,16

Although the level of HDL-C in LA-apoA-I−/− mice did not differ greatly from that in LA-apoA-I+/− mice, HDL in LA-apoA-I−/− mice clearly differed in size and apolipoprotein composition. Similar to what has been reported in apoA-I single knockout mice17 and LDLR/apoA-I double knockout mice,3,18 HDL in LA-apoA-I−/− mice was larger than HDL from LA-apoA-I−/− mice (Figure 1). The HDL peak in LA-apoA-I−/− mice is shifted to the left, corresponding to earlier FPLC fractions that contain larger lipoproteins and that overlap with the LDL peak (Figure 1). Similar to LDLR/apoA-I double knockout mice,3 apoA-II could be detected in earlier FPLC fractions of LA-apoA-I−/− mice than of LA-apoA-I+/− mice (data not shown).

Lipoproteins from LA-apoA-I+/+ and LA-apoA-I−/− mice were isolated by density ultracentrifugation for determination of chemical composition or were subjected to SDS-PAGE and Coomassie staining to characterize the apolipoprotein composition. VLDL and LDL lipid and apolipoprotein compositions were similar in LA-apoA-I+/+ and LA-apoA-I−/− mice (data not shown). Although the total amount of protein and the lipid composition was similar in HDL isolated from LA-apoA-I+/+ or LA-apoA-I−/− mice, the apolipoprotein composition differed (supplemental Figures I and II, available online at http://circres.ahajournals.org). As expected, HDL in LA-apoA-I−/− mice lacked apoA-I and was greatly enriched with apoE compared with HDL from LA-apoA-I+/+ mice.

Atherosclerosis
All mice were fed a standard chow diet. At 3 months of age, all groups were free of lesions, similar to C57BL/6 wild-type mice (data not shown). At 10 months of age, however, an inverse relationship between apoA-I gene dose and lesion area could be observed in both female and male mice (Figure 2A). Both female and male LA-apoA-I−/− mice had a significant 2-fold increase in lesion area compared with LA-apoA-I+/+ mice of the same gender (P<0.0001). LA-apoA-I−/− mice of each gender had a significantly smaller lesion area than their LA-apoA-I+/− counterparts (P<0.01 for female mice, P<0.05 for male mice), but larger lesion area than LA-apoA-I+/+ mice, which was statistically significant for male mice (P<0.0001). An inverse relationship between apoA-I gene dose and lesion stage could also be observed in Oil Red O- and Trichrome-stained aortic root tissue sections in which LA-apoA-I−/− mice had lesions that were the most advanced, whereas LA-apoA-I−/− mice had lesions that were the least advanced (Figure 2B). Complete or partial occlusion of at least one coronary artery could be observed in 6 of 27 LA-apoA-I−/− mice (Figure 2C) compared with partial occlusion in only 1 of 35 LA-apoA-I+/+ mice (P=0.04, Fisher’s Exact Test).

Reverse Cholesterol Transport
We measured the capacity of serum from LA-apoA-I+/+ and LA-apoA-I−/− mice to function as an acceptor of ABCA1- or SR-BI-mediated cellular cholesterol efflux as described previously.6,7 LA-apoA-I−/− serum was a significantly less efficient acceptor of ABCA1-mediated (P<0.05, Figure 3A) and SR-BI-mediated (P<0.0001, Figure 3B) cellular cholesterol efflux than LA-apoA-I+/+ serum. We also observed a marked reduction in LCAT activity in the plasma of LA-apoA-I−/− mice compared with LA-apoA-I+/+ mice (P<0.001; Figure 3C).

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**Table 1. Plasma Lipids in 10-Month-Old Mice**

<table>
<thead>
<tr>
<th></th>
<th>Female</th>
<th>Male</th>
</tr>
</thead>
<tbody>
<tr>
<td>Units mg/dL±SEM</td>
<td></td>
<td></td>
</tr>
<tr>
<td>TC</td>
<td>407±18</td>
<td>413±23</td>
</tr>
<tr>
<td>HDL-C</td>
<td>107±4</td>
<td>97±6</td>
</tr>
<tr>
<td>Non–HDL-C</td>
<td>301±15</td>
<td>316±17</td>
</tr>
<tr>
<td>Triglycerides</td>
<td>71±7</td>
<td>133**±11</td>
</tr>
<tr>
<td>Phospholipids</td>
<td>261±11</td>
<td>277±18</td>
</tr>
</tbody>
</table>

**P<0.001 vs female LA-apoA-I+/+; †P<0.05 vs male LA-apoA-I+/+; ††P<0.001 vs male LA-apoA-I+/+**

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Moore et al ApoA-I and Mechanisms of Atherosclerosis In Vivo 765
To determine whether LA-apoA-I−/− mice had impaired reverse cholesterol transport in vivo, we injected mice intraperitoneally with ac-LDL–loaded J774 macrophage cells labeled with [3H]-cholesterol and measured the appearance of macrophage-derived [3H]-cholesterol in the plasma and feces over a 48-hour period. We observed significantly less macrophage-derived [3H]-cholesterol in the plasma of LA-apoA-I−/− mice than in the plasma of LA-apoA-I+/+ mice (Figure 4A). We also observed significantly less macrophage-derived [3H]-cholesteryl ester in the plasma of LA-apoA-I−/− mice as compared with LA-apoA-I+/+ mice (Figure 4B), consistent with decreased LCAT activity in LA-apoA-I−/− mice (Figure 3C). Compared with LA-apoA-I+/+ mice, an overall defect in macrophage reverse cholesterol transport was apparent in LA-apoA-I−/− mice, in which there was significantly reduced delivery of macrophage-derived [3H]-cholesterol to the feces as either total [3H]-lipid (cholesterol + bile acids) (Figure 4C) or [3H]-cholesterol (Figure 4D) over 48 hours.

**HDL Antioxidant Enzyme Function**

We measured the plasma activity of PON-1 and PAF-AH, 2 enzymes that are HDL-associated in mice and have the ability to hydrolyze inflammatory oxidized phospholipids at the sn2 position.11,12 At 10 months of age, female (Figure 5A) and male (supplemental Figure III) LA-apoA-I−/− mice had a significant ≈75% reduction in PON-1 activity compared with LA-apoA-I+/+ mice (P<0.0001). We measured PON-1 protein mass in the plasma of female mice and observed there to be a less dramatic 39% reduction in PON-1 mass in LA-apoA-I−/− mice compared with LA-apoA-I+/+ mice (P<0.005, Figure 5B). We were therefore able to calculate that there was a 59% decrease in the specific activity of PON-1 in the plasma of female LA-apoA-I−/− mice compared with LA-apoA-I+/+ mice. In LA-apoA-I−/− mice, PON-1 activity was present in FPLC fractions corresponding to the normal HDL cholesterol peak (supplemental Figure IV). Interestingly, in LA-apoA-I−/− mice, PON-1 activity, though markedly reduced, was also present in FPLC fractions corresponding to the normal HDL peak, despite the fact that the majority of the HDL cholesterol present in LA-apoA-I−/− mice is found in FPLC fractions that elute earlier (supplemental Figure IV). No difference in plasma PAH-AH activity was observed between LA-apoA-I+/+ mice and LA-apoA-I−/− mice of either gender (data not shown).

**HDL Antiinflammatory Function**

Lipoproteins from LA-apoA-I−/− and LA-apoA-I+/+ mice were isolated by FPLC to characterize their inflammatory/antiinflammatory potential in a coculture/monocyte chemotaxis assay as described previously.14 HDL from 6- to 8-month-old female and male LA-apoA-I−/− mice had significantly less ability to inhibit monocyte migration than HDL from LA-apoA-I+/+ mice (P<0.005 for female mice, P<0.05 for male mice; Figure 6A). HDL from 6- to 8-month-old male LA-apoA-I−/− mice was proinflammatory in nature, inducing more monocyte migration than when no HDL was added to the coculture (Figure 6A). HDL isolated from 10-month-old male mice yielded similar results (supplemental Figure V). LDL isolated from 6- to 8-month-old mice was proinflammatory in nature, inducing more monocyte migration than LDL from LA-apoA-I+/+ mice (57% increase, P=0.15 in female mice, 120% increase, P<0.05 in male mice; Figure 6B).
LA-apoA-I\(^{−/−}\) mice than in LA-apoA-I\(^{+/+}\) mice (54.9±5.6 pg/mL versus 35.9±5.6 pg/mL, \(P<0.05\); supplemental Figure VII). To determine whether apoA-I–deficient mice had increased sensitivity to an inflammatory challenge, we injected 6-month-old male LA-apoA-I\(^{+/+}\) and LA-apoA-I\(^{−/−}\) mice intraperitoneally with low-dose (12.5 μg) lipopolysaccharide (LPS) and again measured the levels of the same cytokines and chemokines in the plasma 3 and 6 hours after LPS challenge. Three hours after injection of LPS, the levels of interleukin (IL)-6 (\(P<0.005\)) and KC (\(P<0.05\)) were significantly higher in LA-apoA-I\(^{−/−}\) mice than in LA-apoA-I\(^{+/+}\) mice (Table 2). At 6 hours after LPS injection, the levels of tumor necrosis factor-\(\alpha\) (\(P<0.05\)), IL-1\(\beta\) (\(P<0.05\)), IL-2 (\(P<0.05\)), IL-4 (\(P<0.05\)), IL-5 (\(P<0.05\)), IL-10 (\(P<0.05\)), and IL-12 (\(P<0.05\)) were significantly higher in LA-apoA-I\(^{−/−}\) mice than in LA-apoA-I\(^{+/+}\) mice (Table 2). In contrast, the levels of interferon-\(\gamma\), MCP-1, gm-csf, and RANTES were not significantly different between LA-apoA-I\(^{−/−}\) mice and LA-apoA-I\(^{+/+}\) mice at either 3 or 6 hours after LPS injection.

**Discussion**

LDLR/apobec double knockout mice are an ideal model for studying the effects of apoA-I gene dose on the development of atherosclerosis. Chow-fed LA mice have LDL that contains only apoB-100 and a lipoprotein profile that is remarkably similar to many hypercholesterolemic human patients with coronary artery disease. The LA model provides an additional advantage in that in the absence of the LDLR, apoA-I–deficient mice have well-maintained levels of HDL-C. A potential mechanism by which LDLR deficiency increases HDL-C levels in apoA-I–deficient mice is discussed elsewhere. Because HDL-C levels do not differ greatly between groups, mice with different apoA-I genotypes can be compared without major confounding differences in HDL-C, an important factor known to influence the development of atherosclerosis.

We observed an inverse relationship between apoA-I gene dose and atherosclerosis in both female and male mice. To our knowledge, this is the first report of atherosclerosis in apoA-I\(^{−/−}\) mice demonstrating a gene dose effect. Consistent with the increased lesion area in LA-apoA-I\(^{−/−}\) mice, occlusion of coronary arteries also occurred significantly more frequently in LA-apoA-I\(^{−/−}\) mice than in LA-apoA-I\(^{+/+}\) mice. Because differences in HDL-C levels were modest, yet differences in atherosclerosis were pronounced, we attribute much of the difference in atherosclerosis between the groups directly to apoA-I genotype (for additional discussion, please see the online-only data supplement). ApoA-I is the primary protein component of HDL, and apoA-I is also present free in the circulation and interstitial space under varying degrees of lipidation. It is therefore plausible that apoA-I associated with HDL may have direct effects on HDL anti-atherogenic function. Free apoA-I may also have direct effects that are anti-atherogenic, such as functioning as an acceptor of cholesterol and phospholipid efflux from macrophages or removing reactive lipids from apoB-containing lipoproteins.

To investigate the role of apoA-I in the first step in reverse cholesterol transport in which free cholesterol is transported...
out of cells to a cholesterol acceptor, we measured the ABCA1- and SR-BI–mediated cellular cholesterol efflux capacity of serum obtained from mice in our study. ABCA1 functions to transport free cholesterol and phospholipids from cells to an extracellular acceptor.19 Lipid-poor apoA-I is believed to be among the most efficient acceptors of ABCA1-mediated efflux.19 Consistent with this observation, serum from LA-apoA-I/H11002 mice functioned significantly less efficiently as an acceptor of ABCA1-mediated cholesterol efflux from macrophages than serum from LA-apoA-I/H11001 mice. Considerable cholesterol efflux still occurred when LA-apoA-I/H11002 serum was used as an efflux acceptor, however, indicating that apoA-I makes only a relatively minor contribution to the total capacity of serum to function as an acceptor of ABCA1-mediated cholesterol efflux from macrophages, and that other molecules in serum besides apoA-I, such as apoA-II, apoA-IV, and apoE, can also function as acceptors of ABCA1-mediated cholesterol efflux.

In addition to the well-defined role of SR-BI in mediating selective uptake of cholesteryl ester from HDL into cells,20 SR-BI can also function to transport free cholesterol bi-directionally between cells and mature HDL in a concentration-dependent manner.7,19,20 The importance of macrophage SR-BI in the development of atherosclerosis is highlighted by the fact that transplantation of SR-BI/H11002 bone marrow into lethally irradiated apoE/H11002 mice results in significantly increased atherosclerosis.21 SR-BI–mediated cholesterol efflux may therefore be an important mechanism by which excess cholesterol is removed from lesion macrophages. The requirement of apoA-I for efficient SR-BI–mediated uptake of cholesteryl ester from HDL has been demonstrated.22 Here, we now demonstrate that whole serum from LA-apoA-I/H11002 mice also functions less efficiently as an acceptor of SR-BI–mediated free cholesterol efflux. It is likely that reduced SR-BI–mediated cholesterol efflux occurred in LA-apoA-I/H11002 mice because HDL lacking apoA-I is a less efficient acceptor of SR-BI–mediated cholesterol efflux than HDL that contains apoA-I.

Consistent with impaired ABCA1- and SR-BI–mediated cellular cholesterol efflux in vitro, LA-apoA-I/H11002 mice also had significantly reduced transfer of macrophage-derived cholesterol to plasma in vivo. Consistent with the marked
reduction in LCAT activity, LA-apoA-I/H11002/H11002 mice also had significantly reduced accumulation of cholesteryl ester that was derived from 3H-free cholesterol released by macrophages in vivo. Overall, there was significantly reduced net transport of macrophage-derived cholesterol to the liver and feces for excretion in LA-apoA-I/H11002/H11002 mice. These studies highlight the critical role for apoA-I in mediating efficient macrophage reverse cholesterol transport and provide the first in vivo evidence of impaired reverse cholesterol transport in mice lacking apoA-I. These data support the concept that promotion of macrophage reverse cholesterol transport is one mechanism by which apoA-I functions to inhibit the development of atherosclerosis.

We also investigated the role of apoA-I in promoting the activity of the HDL-associated enzymes PON-1 and PAF-AH, which have the ability to hydrolyze oxidized phospholipids.11,12 Reduced plasma PON-1 activity in apoA-I single knockout mice has been reported in one study, and in line (11.1) of human apoA-II transgenic mice that have increased levels of human apoA-II and reduced levels of apoA-I.24 HDL-C levels are reduced by more than 50% in the human apoA-II transgenic mice, however, and are reduced by more than 75% in apoA-I single knockout mice, making it difficult to determine whether the reduction in PON-1 activity is a result of the reduction of apoA-I or HDL-C. Male and female LA-apoA-I−/− mice in our study had only a 14% to 20% reduction in the levels of HDL-C but an ≈75% reduction in total plasma PON-1 activity, indicating that absence of apoA-I rather than reduction of HDL-C was responsible for decreased PON-1 activity. The reduction in total plasma PON-1 activity in LA-apoA-I−/− mice was associated with a decrease in both PON-1 mass and PON-1 specific activity. Although we observed a strong effect of apoA-I genotype on plasma PON-1 activity, we did not observe any effect of apoA-I genotype on PAF-AH activity, indicating that the effects of apoA-I deficiency were specific for PON-1.

Because PON-1–mediated11 and possibly LCAT-mediated25 hydrolysis of proinflammatory oxidized phospholipids is believed to play an important role in HDL antioxidant and antiinflammatory function, and because apoA-I itself is believed to have antioxidant and antiinflammatory effects, we chose to measure HDL antiinflammatory function using an integrated coculture/ monocyte chemotaxis assay.14 Compared with HDL isolated from LA-apoA-I−/− mice, HDL from LA-apoA-I−/− mice had significantly reduced ability to inhibit monocyte migration, and in some cases resulted in the significant reduction of monocyte migration. These data support the concept that promotion of macrophage reverse cholesterol transport is one mechanism by which apoA-I functions to inhibit the development of atherosclerosis.
more monocyte migration than when no HDL was added to the coculture. In addition to impaired HDL antiinflammatory and antioxidant functions in LA-apoA-I\(^{-/-}\) mice, we also observed increased lipid hydroperoxides and inflammatory activity of LDL isolated from LA-apoA-I\(^{-/-}\) mice. Induction of MCP-1 has been shown to be responsible for the majority of the inflammatory activity measured in the coculture/monocyte chemotaxis assay.\(^{26}\) Interestingly, we observed significantly more MCP-1 in the plasma of LA-apoA-I\(^{-/-}\) mice than in LA-apoA-I\(^{+/+}\) mice in vivo, even at 3 months of age, before the onset of atherosclerosis. Taken together, these data suggest that apoA-I is required for normal HDL antiinflammatory function, and that in the absence of apoA-I, both HDL and LDL become more inflammatory, resulting in increased levels of MCP-1, which may in turn promote monocyte migration to within the subintimal space and the development atherosclerosis in LA-apoA-I\(^{-/-}\) mice.

Our LPS injection studies further demonstrate that LA-apoA-I\(^{-/-}\) mice have increased sensitivity to a proinflammatory challenge, indicated by the fact that LPS injection resulted in greater production of several proinflammatory cytokines and chemokines in LA-apoA-I\(^{-/-}\) mice than in LA-apoA-I\(^{+/+}\) mice. Because there were significant differences between LA-apoA-I\(^{-/-}\) and LA-apoA-I\(^{+/+}\) mice in some analytes such as tumor necrosis factor-\(\alpha\), IL-1\(\beta\), and IL-6, whereas other analytes such as MCP-1, gm-csf, and RANTES were induced to a similar level, we consider it unlikely that differences between LA-apoA-I\(^{-/-}\) and LA-apoA-I\(^{+/+}\) mice in some, but not all, analytes measured in LA-apoA-I\(^{-/-}\) mice resulted in greater production of several proinflammatory analytes such as tumor necrosis factor-\(\alpha\), IL-1\(\beta\), and IL-6, whereas other analytes such as MCP-1, gm-csf, and RANTES were induced to a similar level, we consider it unlikely that differences between LA-apoA-I\(^{-/-}\) and LA-apoA-I\(^{+/+}\) mice in some, but not all, analytes measured in LA-apoA-I\(^{-/-}\) mice resulted in greater production of several proinflammatory analytes such as tumor necrosis factor-\(\alpha\), IL-1\(\beta\), and IL-6, whereas other analytes such as MCP-1, gm-csf, and RANTES.

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It is possible that the modest reductions in the levels of HDL-C in LA-apoA-I^{+/−} and LA-apoA-I^{-/−} mice could have contributed to the increase in atherosclerosis in apoA-I deficient mice. However, we observed a 27% increase in atherosclerosis in female heterozygous LA-apoA-I^{+/−} mice compared to LA-apoA-I^{+/+} mice, and 96% increase in atherosclerosis in female LA-apoA-I^{-/−} mice compared to LA-apoA-I^{+/+} mice. These increases are beyond what would be expected due to the modest differences in the levels of HDL-C. Because the levels of non-HDL cholesterol were not significantly different in female LA-apoA-I^{+/+}, LA-apoA-I^{+/−}, and LA-apoA-I^{-/−} mice, we attribute the increase in atherosclerosis to be primarily a result of deficiency of apoA-I.

In male mice there was also a substantially greater increase in the amount of atherosclerosis in apoA-I deficient mice than would be predicted based on differences in the levels of HDL-C alone. However, male LA-apoA-I^{+/−} and LA-apoA-I^{-/−} mice also had somewhat higher levels of non-HDL-C than male LA-apoA-I^{+/+} mice, potentially explaining why apoA-I deficiency was associated with a relatively greater increase in atherosclerosis in male mice (in which apoA-I deficient mice also had increased levels of non-HDL-C) than in female mice (in which non-HDL-C levels were similar between groups).

It is also possible that increased levels of triglycerides may have contributed to increased atherosclerosis in LA-apoA-I^{+/−} and LA-apoA-I^{-/−} mice. However, the increase in triglycerides was quite modest and absolute levels were still fairly low. Other mouse models have been described in which triglycerides are increased, in some cases to a greater extent than in LA-apoA-I^{-/−} mice, without there being any increase in
atherosclerosis. In addition, one school of thought is that triglycerides are a marker for cholesterol-containing, triglyceride-rich lipoproteins, and that the cholesterol-containing lipoproteins are what is thought to be atherogenic. In the FPLC cholesterol profile LA-apoA-I\(^{++}\) and LA-apoA-I\(^{+/-}\) have similar VLDL cholesterol peaks, and the VLDL cholesterol peak in LA-apoA-I\(^{-/-}\) mice is only slightly increased compared to LA-apoA-I\(^{++}\) and LA-apoA-I\(^{+/-}\) mice. Therefore, we consider the effect of increased triglycerides on atherosclerosis to be relatively minor compared to the effects of apoA-I deficiency.

Therefore, although it is possible that differences in plasma lipids may have contributes to increased atherogenesis in apoA-I deficient mice, we estimate this contribution to be relatively minor in comparison to the direct effects of apoA-I deficiency, which include impaired ABCA1 and SR-BI-mediated cholesterol efflux to whole serum, reduced reverse transport of macrophage-derived cholesterol into the plasma and feces for excretion, reduced LCAT activity and reduced esterification of macrophage-derived cholesterol in the plasma, reduced PON-1 activity, and impaired HDL vascular anti-inflammatory activity.

References


Figure I

HDL apolipoprotein composition in 10-month old wild-type, LA-apoA-I$^{+/+}$ and LA-apoA-I$^{-/-}$ mice. HDL was isolated from pooled plasma samples by density gradient ultracentrifugation in the $d=1.063-1.21$ faction. Samples loaded based on equal phospholipid mass were subjected to SDS-PAGE under reducing conditions followed by Coomassie staining. Results are representative of multiple experiments.
Figure II- HDL lipid composition in 10-month old wild-type, LA-apoA-I+/+ and LA-apoA-I−/− mice.
(a) HDL was isolated from pooled plasma samples by density gradient ultracentrifugation in the d=1.063-1.21 faction and percent lipid composition was determined using colorimetric biochemical assays.
Figure III- Plasma PON-1 activity in 10-month old male C57BL/6 wild-type, LA-apoA-I^{+/+}, LA-apoA-I^{+/-}, and LA-apoA-I^{-/-} mice. PON-1 activity was measured in the plasma of 10-month old male mice as described in the methods.

*** p<0.001 vs. LA-apoA-I^{+/+}
Figure IV- Plasma PON-1 FPLC profile in female and male mice. Pooled plasma (4 mice per pool) from 6 to 8-month old female and male LA-apoA-I+/+ and LA-apoA-I-/- mice was fractionated by FPLC and PON-1 activity in each fraction was measured. Values are expressed as the means ± SEM from 3-4 separate plasma pools per group.
Figure V- Monocyte migration assay in 10-month old male mice. LDL was added to cocultures of human artery wall cells in the absence or presence of HDL from 10-month old male LA-apoA-I++/+, LA-apoA-I+/-, and LA-apoA-I-/- mice as described in the methods. Values are expressed as the means ± SEM of the number of migrated monocytes in 9 fields for 3-5 pooled samples per group (3-4 individual mice per pool). Data are normalized to the amount of monocyte migration that occurred when no HDL was added to the cocultures, indicated by the dotted line crossing the y-axis at 1.

* p<0.05 vs. LA-apoA-I++/
# p<0.005 vs. LA-apoA-I+/-
Figure VI- LDL-associated lipid hydroperoxides in 6 to 8-month old female and male La-apoA-I+/+ and LA-apoA-I-/- mice. LDL was isolated from female and male LA-apoA-I+/+ and LA-apoA-I-/- mice by FPLC. LDL associated lipid hydroperoxides were determined by the method of Auerback in which N-benzoyl leucomethylene blue is oxidized to methylene blue by lipid hydroperoxides present in the sample. LDL-associated lipid hydroperoxides are normalized to the amount of LDL protein in each sample.
Figure VII- Plasma levels of MCP-1 in 3-month old female LA-apoA-I+/+ and LA-apoA-I-/- mice. The level of MCP-1 protein in the plasma of 3-month old female LA-apoA-I+/+ and LA-apoA-I-/- mice was determined using multiplex sandwich ELISA (Pierce Biotechnology).