Overexpression of Apolipoprotein A-I Promotes Reverse Transport of Cholesterol From Macrophages to Feces In Vivo

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Background—Abundant data indicate that overexpression of apolipoprotein A-I (apoA-I) in mice inhibits atherosclerosis. One mechanism is believed to be promotion of reverse cholesterol transport, but no direct proof of this concept exists. We developed a novel approach to trace reverse transport of labeled cholesterol specifically from macrophages to the liver and feces in vivo and have applied this approach to investigate the ability of apoA-I overexpression to promote macrophage-specific reverse cholesterol transport.

Method and Results—J774 macrophages were loaded with cholesterol by incubation with acetylated LDL, labeled with $^3$H-cholesterol, and then injected intraperitoneally into mice. Plasma and feces were collected at 24 hours and 48 hours, when mice were exsanguinated, tissues were harvested, and all were analyzed for tracer counts. $^3$H-cholesterol was found in the plasma, liver, and feces. For apoA-I overexpression, mice were injected intravenously with apoA-I adenovirus (10¹¹ particles per animal) 3 days before labeled macrophages were injected. ApoA-I overexpression led to significantly higher $^3$H-cholesterol in plasma, liver, and feces. The amount of $^3$H-tracer in the liver was 35% higher ($P<0.05$) and the $^3$H-tracer excreted into feces over 48 hours was 63% higher ($P<0.05$) in apoA-I–expressing mice than in control mice.

Conclusion—Injection of $^3$H-cholesterol–labeled macrophage foam cells is a method of measuring reverse cholesterol transport specifically from macrophages to feces in vivo, and apoA-I overexpression promotes macrophage-specific reverse cholesterol transport. (Circulation. 2003;108:661-663.)

Key Words: cholesterol ■ cholesterol, reverse transport ■ apolipoproteins ■ macrophages ■ atherosclerosis

There is a strong inverse association between plasma HDL cholesterol levels and incidence of atherosclerotic cardiovascular diseases. Apolipoprotein A-I (apoA-I) is the major protein in HDL and is synthesized and secreted by the intestine and the liver. Abundant data from studies in animals indicate that hepatic overexpression of apoA-I inhibits atherosclerosis, but the mechanism(s) remain uncertain. ApoA-I is thought to protect against atherosclerosis at least in part by promoting efflux of excess cholesterol from macrophages in the arterial wall and returning that cholesterol to the liver for excretion into the bile, a process known as “reverse cholesterol transport.” There have been various efforts to quantify reverse cholesterol transport in animals in response to changes in apoA-I levels. Stein et al reported no evidence of increased rate of loss of $^3$H-cholesterol from a muscle depot in human apoA-I–transgenic mice. Dietsch and colleagues reported that there was no difference in the rate of “net centripetal cholesterol flux” from the peripheral tissue under 2-fold difference in plasma apoA-I and thatapoA-I–knockout mice were not different from wild-type mice with regard to peripheral cholesterol efflux. By measuring centripetal cholesterol flux and fecal sterol excretion, Spady and colleagues found no increase in reverse cholesterol transport in mice overexpressing apoA-I after injection of a recombinant adenoviral vector.

Therefore, despite continued enthusiasm for the concept of reverse cholesterol transport as a major mechanism by which apoA-I overexpression protects against atherosclerosis, no direct proof of this concept yet exists, which has created substantial doubt as to whether HDL and apoA-I actually promote the rate of reverse cholesterol transport. However, the methods used previously have estimated rates of reverse cholesterol transport from entire peripheral tissue and not specifically from macrophages, the most important cholesterol-accumulating cells in atherosclerosis. Macrophages are particularly reliant on the ABCA1 pathway, which is promoted by apoA-I, to rid themselves of excess cholesterol. We therefore developed a novel approach to measure reverse...
transport of labeled cholesterol specifically from macrophages to the liver and feces in vivo and have applied this approach to investigate the ability of apoA-I overexpression to promote macrophage-specific reverse cholesterol transport.

### Methods

#### J774 Cell Culture, 3H-Cholesterol Labeling, and Cholesterol Loading

J774 cells were obtained from American Type Culture Collection (ATCC; Manassas, Va) and were grown in suspension in RPMI 1640 supplemented with 10% fetal bovine serum. Cells were radiolabeled with 5μCi/mL 3H-cholesterol and cholesterol enriched with 100 μg/mL of acetylated LDL for 48 hours. These foam cells were washed twice, equilibrated in medium with 0.2% bovine serum albumin for 6 hours, spun down, and resuspended in 0.5 mL medium. The distribution of 3H-cholesterol between free cholesterol and cholesterol ester in J774 cells was determined by thin-layer chromatography (TLC). Cholesterol mass was determined by gas liquid chromatography (GLC). The cholesterol content of J774 foam cells was markedly elevated, and the majority of cellular cholesterol was esterified. The distribution between free and esterified cholesterol was consistent in both TLC and GLC assays.

#### In Vivo Studies

Twenty male C57BL/6 mice (Jackson Laboratories, Bar Harbor, Me) were injected intravenously with recombinant adenoviral vector encoding human apoA-I (AdapoA-I, n=10) or a control adenovirus containing no transgene (Adnull, n=10) (1010 particles per animal). Three days after vector injection, 3H-cholesterol–labeled J774 foam cells (7.8×106 cells containing 3.8×106 counts per minute [cpm] in 0.5 mL minimum essential medium) were injected intraperitoneally. Blood was collected at 24 hours (retro-orbital plexus) and 48 hours and were stored at −20°C until extraction of cholesterol and bile acid. At 48 hours, mice were anesthetized, and the vasculature was perfused with cold phosphate-buffered saline. Liver, spleen, and lungs were removed and stored at −20°C until lipid extraction. An additional apoA-I overexpression experiment was performed in C57BL/6 female mice (n=8; Jackson Laboratories).

#### Fecal Cholesterol and Bile Acid Extraction

Fecal cholesterol and bile acid were extracted as described previously. The levels are expressed as counts per minute in total feces by wet weight.

#### Tissue Lipid Extraction

Tissue lipids were extracted using the method of Bligh and Dyer and expressed as counts per minute in total organ. The distribution of cholesterol between free and esterified forms in liver, spleen, and lung were measured by both TLC and GLC.

#### Plasma Lipid Analysis

Plasma total cholesterol, HDL cholesterol, phospholipid, and human apoa-I levels were measured on a Cobas Fara (Roche Diagnostics Systems, Inc) using Sigma Diagnostics reagents as described previously, and the levels were expressed as milligrams per liter. Serum lipoproteins were isolated by ultracentrifugation and analyzed for the distribution of 3H-cholesterol among lipoprotein fractions.

#### Statistical Analysis

Values are presented as mean±SEM. Results were analyzed by ANOVA and Student’s t test with the use of GraphPad Prism Software.

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**Figure 1.** C57BL/6 mice were injected intravenously with apoA-I adenovirus (n=10) or control (null) adenovirus vector (n=10) (1.0×1011 particles of each virus per animal). Three days after vector injection, 3H-cholesterol–labeled J774 foam cells were injected (7.8×106 J774 cells containing 3.8×106 cpm 3H-cholesterol in 0.5 mL medium). A, 3H-tracer in plasma at 24 and 48 hours. B, 3H-tracer in tissue and feces.

**Figure 2.** 3H-cholesterol and 3H-bile acids in feces at 24 hours and 48 hours after overexpression of apoA-I in mice injected with 3H-cholesterol–labeled J774 foam cells.
control mice (Figure 1B). The $^3$H-tracer detected in the fecal bile acid was similar in both apoA-I–overexpressing mice and control mice at 24 hours and 48 hours (Figure 2). In a separate experiment, overexpression of apoA-I in female mice resulted in increases in $^3$H tracer in plasma, tissues, and feces similar to those observed in the male mice (data not shown).

**Discussion**

We report the development of a novel approach for tracing reverse transport of radiolabeled cholesterol specifically from macrophages to the liver and feces in vivo. After intraperitoneal injection of $^3$H-cholesterol–labeled macrophage foam cells, $^3$H-cholesterol was detected in plasma, lung, spleen, liver, and feces. Furthermore, a substantial fraction of the tracer in feces was found in bile acids, indicating conversion of $^3$H-cholesterol into $^3$H-bile acid in the liver. These results demonstrate that $^3$H-cholesterol originating in cholesterol-loaded macrophages was transported through the plasma to the liver, converted in part into bile acids, and then excreted as either free cholesterol or bile acid into bile and ultimately into the feces. Mice overexpressing apoA-I had significantly higher plasma $^3$H-cholesterol and higher $^3$H-tracer in the liver and excreted 63% more $^3$H-tracer into feces over 48 hours than did control mice.

Although we cannot fully rule out the migration of intact macrophages from the peritoneum to the liver, an examination of the distribution of $^3$H-cholesterol between free and esterified pools speaks against this. In our pilot experiment, we measured the distribution of free and esterified cholesterol in $^3$H-cholesterol–labeled J774 foam cells and in mouse liver, spleen, and lung by TLC and also determined their masses of free and esterified cholesterol. The majority of cellular cholesterol of labeled J774 foam cells was esterified ($\approx$70%), whereas $\approx$15%, 13%, and 11% of the $^3$H-cholesterol in the liver, spleen, and lung was esterified. This suggests that the $^3$H-sterol in tissues was not transported there by macrophages. The abundant $^3$H-cholesterol detected in plasma and the fact that 75% of the tracer in plasma was in the HDL fraction suggests a model in which $^3$H-cholesterol was available for efflux from macrophages in situ and was transported through the plasma compartment to the liver, mainly on HDL particles.

It has been widely assumed that apoA-I overexpression inhibits atherosclerosis at least in part by reverse cholesterol transport. However, data proving this have been lacking, raising the question as to whether apoA-I overexpression actually promotes the rate of reverse cholesterol transport. The methods used previously have estimated rates of reverse cholesterol transport from entire peripheral tissue and not specifically from macrophages. Our studies demonstrate for the first time that apoA-I overexpression promotes macrophage-specific reverse cholesterol transport in vivo. It is possible that macrophages are more responsive to increases in plasma concentrations of apoA-I than are other cell types, and this tracer approach may allow more effective measurement of reverse cholesterol transport than mass-based approaches.

In summary, we report the development of a novel approach for tracing reverse transport of labeled cholesterol specifically from macrophages to the liver and feces in vivo. Using this approach, we demonstrate for the first time that apoA-I overexpression promotes macrophage-specific reverse cholesterol transport in vivo. This method may be applied to other questions about the roles of specific genes in regulating the rate of reverse cholesterol transport.

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**References**

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