Red Blood Cells Play a Role in Reverse Cholesterol Transport

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Objective—Reverse cholesterol transport (RCT) involves the removal of cholesterol from peripheral tissue for excretion in the feces. Here, we determined whether red blood cells (RBCs) can contribute to RCT.

Methods and Results—We performed a series of studies in apolipoprotein AI-deficient mice where the high-density lipoprotein–mediated pathway of RCT is greatly diminished. RBCs carried a higher fraction of whole blood cholesterol than plasma in apolipoprotein AI-deficient mice, and as least as much of the labeled cholesterol derived from injected foam cells appeared in RBCs compared with plasma. To determine whether RBCs mediate RCT to the fecal compartment, we measured RCT in anemic and control apolipoprotein AI-deficient mice and found that anemia decreased RCT to the feces by over 35% after correcting for fecal mass. Transfusion of [3H]cholesterol–labeled RBCs led to robust delivery of the labeled cholesterol to the feces in apolipoprotein AI-deficient hosts. In wild-type mice, the majority of the blood cholesterol mass, as well as [3H]cholesterol derived from the injected foam cells, was found in plasma, and anemia did not significantly alter RCT to the feces after correction for fecal mass.

Conclusion—The RBC cholesterol pool is dynamic and facilitates RCT of peripheral cholesterol to the feces, particularly in the low high-density lipoprotein state. (Arterioscler Thromb Vasc Biol. 2012;32:1460-1465.)

Key Words: apolipoprotein A-I ■ high-density lipoprotein ■ lipids ■ lipoproteins ■ macrophages

It is generally accepted that lipoproteins carry cholesterol in plasma throughout the body. Increasing high-density lipoprotein cholesterol (HDL-C) via apolipoprotein AI (apoAI) gene transfer in mice has been shown to increase reverse cholesterol transport (RCT), as measured by tracing the levels of [3H]cholesterol transferred from injected macrophages to the plasma, liver, and feces. Yet, in humans, whole blood is composed of ≈45% red blood cells (RBCs) by volume, and the cholesterol concentration in RBCs is comparable to that found in the plasma, carried by lipoproteins. RBC plasma membranes contain free cholesterol that can bidirectionally exchange with plasma lipoprotein cholesterol approaching equilibrium ex vivo in ≈6 hours, with the kinetics indicating transfer via aqueous diffusion. Despite their significant carrying capacity for cholesterol, the role that RBCs may play in RCT has not been previously addressed. Here we show that apoAI-deficient (apoAI−/−) mice carry most of their cholesterol in the RBC compartment as opposed to the plasma compartment. When we made these apoAI−/− mice anemic, RCT to the fecal compartment was reproducibly decreased.

Methods

Mice

Wild-type (WT), apoAI−/−, and apoAI transgenic mice, all on the C57BL/6 background, were purchased from The Jackson Laboratory.

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RBC dpm was calculated by estimating blood volume to be equal to 7% of the body weight and RBC volume to be 45% of the blood volume, or the value based on the observed hematocrit. RCT to the RBC compartment was quantified as the percentage of dpm in total RBCs/ injected dpm. Feces were collected daily and allowed to soften in a 50% ethanol solution. In certain experiments, feces collected daily were first dried overnight at 55°C, weighed, and then softened in 50% ethanol solution. After hydration, the feces were homogenized, and an internal recovery standard of 10 000 dpm of [14C]cholesterol (Perkin Elmer) was added to each sample. The radioactivity in a 0.3-mL aliquot of the fecal homogenate was measured by liquid scintillation counting after chemiluminescence had decayed. The [14C]cholesterol dpm was used to back calculate the [H] recovery for the entire fecal homogenate. RCT to the feces was calculated as the percentage of dpm in feces/injected dpm. We have observed that fecal RCT can be artificially high for some mice if any of the injected labeled cells leak out of the injection site and are consumed orally by the mice. Thus, any mouse with fecal RCT >2 SDs above the mean of the remaining data within each group was excluded from analysis. Fecal neutral sterols were analyzed by modification of a previously described protocol. Sterols were saponified in 0.3 mL aliquots of fecal homogenates by adding 0.3 mL of ethanol and 0.4 mL of 1M NaOH. After heating at 95°C for 2 hours, neutral sterols were extracted 3x with 9 mL hexane, pooled, dried, and counted. The bottom phase was adjusted to neutral pH and counted as the bile acid fraction. Average recovery of the [14C]cholesterol internal standard in the neutral sterol fraction was 96.5±6.2%. Upon euthanization, each mouse was perfused with PBS by making an incision in the right atrium and injecting the left ventricle with 10 mL of PBS. The liver was harvested, weighed, suspended in PBS, homogenized, and 10 000 dpm of [14C]cholesterol was added as a recovery standard, with aliquot counting and RCT calculation as described above.

Plasma and RBC Cholesterol Assays
Whole blood was centrifuged at 305 x g for 5 minutes to separate the plasma from cellular compartments. For simplicity, we refer to the cellular fraction as the RBC compartment, although it also contains leukocytes and platelets. The plasma cholesterol concentration was determined using an enzymatic assay from StanBio Laboratory. The RBC fraction was carefully washed twice in PBS to remove any plasma contamination, and lipids in the cell pellet were extracted with hexane:isopropyl alcohol (3:2). After drying the solvent, lipids were dissolved in isopropanol:nonyl phenoxypolyethoxylethanol-40 (9:1), and cholesterol was measured as previously described. The concentrations of the plasma and cellular cholesterol pools were normalized to 1 dL of whole blood assuming that the cellular and plasma fractions make up 45% and 55%, respectively, of whole blood volume.

Induction of Anemia
ApoAI−/− or WT mice were anesthetized with isoflurane and bled retro-orbitally into a heparinized capillary tube for 3 consecutive days (≈290 μL of blood per day) to induce hemorrhagic anemia. In specified studies, the blood was centrifuged to isolate the plasma, and then the plasma was re-injected retro-orbitally with a 26-gauge needle into the contralateral retro-orbital plexus while the mouse was under anesthesia. An additional 60 μL of blood was removed retro-orbitally to determine daily hematocrits. Thus, a total of ≈350 μL of blood was bled from each mouse in the anemic group daily. Control mice were anesthetized with isoflurane and then allowed to recover without bleeding. The anemic and control mice were used for RCT studies as described above.

Ex Vivo Labeling and Transfusion of RBCs
A WT mouse was bled bident-orbitally to collect 1 mL of whole blood. The blood was spun at 400g for 15 minutes, and the plasma anduffy coat layers removed. To label RBCs, the RBC pellet was resuspended to 1 mL of PBS and incubated with 10 μCi [1H]cholesterol (1% ethanol final concentration) for 10 minutes at 37°C. The RBCs were washed twice by suspension in PBS and centrifugation at 400g for 15 minutes, resuspended into 1.5 mL PBS, and injected (100 μL per mouse) retro-orbitally into each recipient mouse while the mouse was anesthetized with isoflurane. At ≈1 minute after injection, blood was obtained from the tail vein to calculate injected radioactivity at time zero. After 48 hours, the recovery of [H] radioactivity and calculation of the percentage of the time zero dose in the plasma, RBCs, liver, and feces were performed as described above.

Results and Discussion
A series of observations led us to hypothesize the existence of a novel non-HDL pathway involving RBCs that contributes to fecal RCT. First, RCT was assessed in murine models by subcutaneous injection of cholesterol-labeled foam cells and following the cholesterol radioactivity into the plasma, hepatic, and fecal compartments over 3 days. RCT was compared in apoAI−/−, WT, and human-apoAI transgenic mice, with varying levels of HDL-C (12.2±13.5, 92.9±10.5, and 135.7±12.1 mg/dL, respectively; P<0.001 by ANOVA post-test). Compared with apoAI transgenic hosts, apoAI−/− hosts had ≈18-fold less [H]cholesterol transfer to the plasma but only 2.2-fold less transfer to the feces (Figure 1A). Thus, in apoAI−/− mice, where HDL-mediated RCT is greatly diminished, RCT to the feces was relatively preserved. Secondly, we examined the steady-state cholesterol content in whole blood and found that the plasma and RBC (including other minor cellular fractions) cholesterol pools were roughly equivalent in WT mice. However, in apoAI−/− mice, the RBC compartment had >2-fold higher cholesterol level than the plasma compartment (Figure 1B). Thirdly, we followed [H] cholesterol appearing in both the plasma and RBC compartments in an RCT study of apoAI−/− hosts. Over 4 days, we observed a trend that more of the cholesterol tracer was found in the RBC versus the plasma pool (Figure 1C), implicating a role for RBCs in RCT. Based on these initial observations, we hypothesized the existence of an alternative non-HDL pathway by which peripheral cholesterol reaches the feces, involving RBCs, the other major reservoir of cholesterol in blood.

RBCs serving as a non-HDL pathway for RCT would be especially evident in apoAI−/− mice because they carry a greater fraction of cholesterol in the RBC compartment versus the plasma compartment compared with WT mice. Thus, decreasing the quantity of RBCs in apoAI−/− mice would be expected to decrease the transfer of [H]cholesterol from foam cells to the feces. To test this hypothesis, we induced hemorrhagic anemia in apoAI−/− female mice by removing ≈0.3 mL of blood from the retro-orbital plexus for 3 consecutive days, and on the third day (day 0 of the RCT assay in Figure 2A) cholesterol-labeled macrophages were injected subcutaneously into anemic and control apoAI−/− mice. Blood was drawn from each mouse on 2 subsequent days to determine hematocrits as well as the RCT to the plasma and RBC compartments. Feces were collected daily, and perfused livers were harvested on day 2. On days 1 and 2 of the RCT study, the hematocrits of the anemic mice were significantly lower than the control mice (Figure 2A, P<0.001). The decreased hematocrit of the control mice on day 2 versus day 1 was significant (P<0.01) and reproducible in response to the diagnostic bleeding on day 1. The increase in hematocrits in the anemic mice on day 2 versus day 1 (P<0.001) was reproducible, and presumably

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reflects increased erythropoiesis induced by mild hypoxia that can overcome the small blood loss due to diagnostic bleeding on day 1. RCT to the plasma pool was low in both groups and not altered by anemia on either day (Figure 2B). RCT to the RBCs was higher in the control group than the anemic group on both days ($P<0.05$), reflecting the smaller RBC pool in anemic mice. The sum of the RCT to the plasma and RBC compartments, namely RCT to whole blood, was reduced by 49% on day 1 and 25% on day 2. Anemia had no statistically significant effect on RCT to the liver, although there was a trend toward lower hepatic RCT in the anemic group. Cumulatively over 2 days, RCT to the feces was reduced by 54% in the anemic mice ($P<0.01$). We repeated the anemia experiment in male apoAI$^{-/-}$ mice and observed similar effects with a 38% decrease in fecal RCT ($P<0.05$) and no significant effect on hepatic RCT (data not shown). The experiment was repeated again in female apoAI$^{-/-}$ mice with 2 modifications: plasma removed during bleeding to induce anemia was reinjected intravenously to restore plasma protein, and dried fecal weights were determined to ensure that decreased fecal RCT in the anemic group was not due to decreased fecal output. Anemia led to a 58% decrease in RCT over 2 days ($P<0.01$), no significant effect on hepatic RCT, but there was also a 33% decrease in dried fecal weight ($P<0.05$), which we attribute to the observed lethargy and presumed lower food consumption in the anemic mice. After normalizing RCT to the dried fecal mass, anemia still led to a 35% decrease in RCT/g feces ($P<0.05$). Thus, the decreased RBC pool size in anemic mice decreased RCT to the feces independent of any effects on decreased fecal output.
Our observation that anemia greatly impairs RCT to the feces without a significant effect on RCT to the liver suggests that RBCs may deliver their cholesterol to the feces by >1 route, such as the direct transintestinal cholesterol efflux pathway, or that cholesterol delivered to the liver by RBCs versus HDL is handled differently in the liver. Alternatively, anemia may modestly impair RCT to the liver, but this difference may be too small to be detected without more power. To determine whether RBC cholesterol is efficiently transferred to the feces, we labeled mouse RBCs ex vivo with [3H]cholesterol and transfused them into apoAI−/− mice. Radioactivity in the RBCs, plasma, liver, and feces was measured 2 days later. Only 4.5% of the injected cholesterol tracer remained in the RBC fraction, demonstrating rapid turnover of this pool, and even less (1.3%) was found in the plasma (Figure 3A). Most of the radioactivity was recovered in the liver (21.5%) and feces (32%). The observation that the feces and liver contain more of the tracer than the rest of the carcass is remarkable because it demonstrates highly robust transfer of RBC cholesterol to the liver and feces despite diminished HDL, which is thought to mediate much of the hepatobiliary RCT pathway. To determine whether the transintestinal cholesterol efflux pathway might play a prominent role in the delivery of RBC cholesterol to the feces, we compared the fraction of fecal [3H] cholesterol in neutral sterols (because direct intestinal cholesterol secretion would increase the fraction in neutral sterols versus bile acids) between WT mice (where plasma and RBC cholesterol pools are similar) and apoAI−/− mice (where RBCs constitute the major blood cholesterol pool). We found that the neutral sterol [3H] fraction was 20.7±3.1% in WT mice, but only 13.0±2.4% in apoAI−/− mice (P<0.05, Figure 3B). We observed a compensatory change for the [3H] recovered in the bile acid fraction with 80.2±2.2% in WT mice and 88.6±1.9% in apoAI−/− mice (P<0.01). Thus, in apoAI−/− mice where the majority of the RCT [3H]cholesterol pool of whole blood is in RBCs, this cholesterol is more efficiently converted into bile acids compared with WT mice, suggesting more efficient bile acid synthesis from the RBC cholesterol pool versus the HDL-C pool. Combined with the finding of robust transfer of RBC-derived cholesterol tracer to the liver, our data support the model that RBC cholesterol traffics through the liver more efficiently than HDL-C, whose uptake and metabolism are dependent on scavenger receptor class B type I trafficking. Furthermore, our data suggest that the transintestinal pathway does not play a prominent role in RCT mediated through the RBC cholesterol pool.

To determine whether anemia could modulate RCT in mice with normal HDL-C levels, we repeated the anemia study previously done on apoAI−/− hosts, but this time in WT male mice. We induced anemia by bleeding on days −2 to 0 of the anemia time course, and the plasma recovered from whole blood was reinjected intravenously (n=7 each in the control and anemic groups). On days 1 and 2 of the RCT study, the hematocrits of the anemic mice were significantly lower than the control

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**Figure 3.** A, Yields of radioactivity in various compartments 2 days after intravenous transfusion of [3H]cholesterol-labeled red blood cells (RBCs) into apolipoprotein Al-deficient (apoAI−/−) hosts (n=4±SD). B, Percentage of fecal [3H] in neutral sterols from feces collected on day 2 after subcutaneous injection of [3H] cholesterol-labeled foam cells into wild-type (WT) and apoAI−/− hosts (n=3 WT and 4 apoAI−/−, ±SD; *P<0.05 vs WT).

**Figure 4.** A, Hematocrits of wild-type mice throughout the reverse cholesterol transport (RCT) assay (n=7±SD; *P<0.001 vs controls by 2-tailed t test). B, RCT to various compartments in anemic and control mice along with fecal weight (right axis) and fecal RCT adjusted to fecal weight (***P<0.001 vs control).
mice (Figure 4A, P<0.001). RCT to the plasma pool in WT mice was robust, compared with apoAI−/− mice, and not altered by anemia on either day (Figure 4B). RCT to the RBCs was higher in the control group than the anemic group on both days (P<0.001), reflecting the smaller RBC pool in anemic mice. RCT to whole blood, the sum of RCT to the plasma and RBC compartments, was reduced by 20% on day 1 and only 7% on day 2 (compared with 49% and 25% reductions on days 1 and 2, respectively, in the experiment performed in apoAI−/− deficient hosts shown in Figure 1B), reflecting the larger proportion of RCT mediated through the plasma (lipoprotein) compartment. Anemia had no statistically significant effect on RCT to the liver. Cumulatively over 2 days, RCT to the feces was reduced by 39% in the anemic mice (Figure 4B, P<0.001). However, this effect was due to the significantly lower fecal mass in the anemic group (P<0.001), because after correction for fecal mass, there was only a nonsignificant trend with 17.4% reduced fecal RCT in the anemic group (Figure 4B). A power analysis based on these data demonstrated that we would need ≈30 mice per group to have 80% power to detect this effect at P<0.05. Because mice are by nature a high HDL species, it is not surprising that anemia did not have a significant impact on total fecal RCT in WT mice over the 2-day time course. On further examination, we found that RBCs accounted for 53.4% and 59.9% of whole blood RCT in apoAI−/− mice (day 1 and day 2, respectively), while only accounting for 9.6% and 12.1% of whole blood RCT in WT mice (Figures 2B and 4B).

Here we show that the RBC cholesterol pool may play a previously unknown role in mediating RCT. We propose a model (Figure 5) in which interstitial apoAI, apoE, HDL, and low-density lipoprotein can accept or exchange foam cell cholesterol and deliver it to the blood stream. In our studies in apoAI−/− mice, we suggest that lipid-free apoE or other exchangeable apolipoproteins can pick up free cholesterol and phospholipids to form nascent HDL by interacting with ABCA1 on the foam cells. ApoE-containing HDL as well as low-density lipoprotein can also pick up or exchange with [3H] cholesterol in the foam cells and carry it into the blood stream. In whole blood, the free cholesterol pool can passively equilibrate between lipoproteins and cell membranes, with RBCs providing the bulk of cell membranes. Although cholesteryl ester transfer protein is not expressed in mice, it may contribute to cholesterol ester exchange from HDL to low-density lipoprotein in humans and other species where it is expressed. When RBCs pass through the liver, they can deliver their cholesterol to sinusoidal endothelial cells which in turn can pass it to hepatocytes. Whether this step is entirely passive or may be facilitated by a transporter is unknown. Alternatively, cholesterol transfer from RBCs to hepatocytes may again be mediated via exchange to lipoproteins that can then migrate into the space of Disse and deliver cholesterol to hepatocytes.

**Figure 5.** Working model for the role of red blood cells (RBCs) in reverse cholesterol transport (RCT). Interstitial apolipoprotein E (apoE), apoAI, high-density lipoprotein (HDL), and low-density lipoprotein (LDL) can accept or exchange free cholesterol from foam cells and enter the blood stream. Lipoprotein-free cholesterol can then be transferred to RBC plasma membranes. RBCs transit to the liver where they can donate free cholesterol directly to sinusoidal endothelial cells that are in contact with hepatocyte projections, or can deliver cholesterol to hepatocytes indirectly through cholesterol transfer to lipoproteins that can enter the space of Disse. Illustration by David Schumick, BS, CMI. Reprinted with the permission of the Cleveland Clinic Center for Medical Art & Photography © 2012. All Rights Reserved. CETP indicates cholesteryl ester transfer protein; VLDL, very low-density lipoprotein.
However, our finding of a smaller fraction of RCT-derived fecal neutral sterols in apoAI−/− versus WT mice supports the notion that the transfer of RBC cholesterol to hepatocytes may at least partially be independent of lipoproteins. Once in the hepatocyte, most of the RBC-derived free cholesterol is efficiently converted to bile acids, which along with the remaining free cholesterol is excreted into the bile and then into the intestine.

In a longitudinal study of 14,410 human subjects, anemia at baseline was associated with a significant hazard ratio of 1.41 for subsequent coronary vascular disease over a 6-year follow-up. Although the mechanism for this association is unknown, our findings suggest that diminished RCT in anemic subjects may play a role in this association. Low HDL-C levels are common in men, and men with 35 mg/dL have HDL levels closer to those seen in apoAI−/− mice (12 mg/dL) than seen in WT mice (93 mg/dL). Thus, anemia and hematocrits should not be overlooked in ongoing clinical studies of RCT and HDL function.

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Disclosures
None.

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