In Vivo Reverse Cholesterol Transport From Macrophages Lacking ABCA1 Expression Is Impaired

Ming-Dong Wang, Vivian Franklin, Yves L. Marcel

**Background**—ATP-binding cassette transporter A1 (ABCA1) is a key mediator of cholesterol efflux to apoA-I in cholesterol loaded macrophages, a first step of reverse cholesterol transport (RCT) in vivo. Macrophage specific abca1 inactivation or overexpression, respectively, accelerated or suppressed the development of atherosclerosis in mouse models. However, it is yet to be established that the ABCA1 effect is related to specific changes in RCT from the macrophage in vivo.

**Methods And Results**—Bone marrow–derived macrophages from abca1−/− or abca1+/− mice were labeled with 3H-cholesterol-AcLDL or 3H-cholesterol-LDL and injected into abca1+/+ abcg1+/+ or abca1−/− mice. When injected into abca1−/− mice, return of 3H-cholesterol from labeled abca1−/− macrophages to serum, liver, bile, and feces was reduced by 50% (P=0.01) compared with control. When labeled wild-type macrophages were injected into abca1−/− mice, as compared with wild-type mice, the return of 3H-cholesterol to serum, liver, bile, and feces was also reduced.

**Conclusions**—ABCA1 expression in macrophages contributes significantly to in vivo macrophage RCT. The important residual RCT observed from abca1−/− macrophages highlight the functionality of transporters that efflux to HDL.

*(Arterioscler Thromb Vasc Biol. 2007;27:1837-1842.)*

**Key Words:** lipoproteins ■ HDL ■ monocyte-derived macrophage

The concept of reverse cholesterol transport (RCT) was first proposed by Glomset in 1968,1 but it remains to be firmly established that RCT is the main atheroprotective property of HDL. RCT refers to the process by which cholesterol from the peripheral tissues is transported via plasma high-density lipoprotein (HDL) to the liver for excretion as cholesterol or acidic sterol into bile and eventually the feces. Individual steps of RCT have been extensively defined and validated in vitro and in vivo. However, the in vivo validation of the pathway and the evaluation of its regulation at different steps have lagged behind. Recently, Rader and colleagues have described an in vivo method and model for the study of RCT.2,3 J774 cells were loaded with 3H-cholesterol-labeled acetylated LDL and injected intraperitoneally into recipient mice. Subsequent collection of liver, gall bladder, and feces demonstrated effective RCT by a process enhanced by overexpression of apoA-I. This method has for the first time allowed in vivo analysis of the efficiency of individual steps and factors involved in this complex pathway. Follow-up studies demonstrated the importance of SR-BI as a rate-controlling receptor for hepatic uptake of HDL cholesterol1 and activation of LXR with GW3965 enhanced in vivo RCT in all mouse models,4 thus providing proof of principle that this method can be used to monitor changes in RCT and linked to changes in atherosclerosis burden.

A number of pathways contribute to efflux of lipids from macrophages (reviewed in5–9). ABCA1 effluxes lipids to lipid-poor apolipoproteins, mainly apoA-I, whereas ABCG1 and SR-BI efflux cholesterol to HDL, but the respective importance of these 3 transporters in vivo is not known. Other pathways operating by diffusional mechanisms through cholesterol gradients also appear significant but remain uncharacterized.10 ABCG4 is another macrophage transporter which, like ABCG1, transfers cholesterol to HDL,11,12 but is still poorly characterized. ApoE secretion represents a significant pathway for lipid export from macrophages which is independent of ABCA1,13 but secreted apoE can interact with macrophage ABCA114 and this pathway is enhanced by SR-BI15 and by ABCG1 inactivation.16 The contribution of the diverse macrophage efflux pathways to RCT is still not known. A recent study that analyzed the effect of ABCA1 levels simultaneously in macrophage and liver could not differentiate between the direct contribution of ABCA1 to efflux and its effect on efflux acceptors, because of the decreased HDL levels.17 Targeted inactivation of abca1 has provided data on the contribution of specific organs to the circulating levels and pool of HDL providing estimates of about 20% for macrophages,18,19 80% for the liver,20 and 20% for intestine.21 Liver ABCA1 contributes to the lipiddation of newly synthesized and secreted apoA-I and thus export
cholesterol and other lipids via HDL, and although the secreted lipoproteins are not fully lipidated,22–24 this initial lipidation is essential to maintain the nascent HDL in circulation.25

Here we have evaluated the effect of abca1 inactivation in macrophages and in the whole animal on RCT from macrophages labeled and loaded with either AcLDL or LDL. We demonstrate that macrophage ABCA1 activity accounts for about half of the transfer of label to liver, bile, and feces.

Methods and Materials

Animals

abca1+/− mice (in C57 background) were a kind of gift from Dr Edward M. Rubin, DOE Joint Genome Institute, Berkeley, Calif. To increase the breeding efficiency of abca1+/− male abca1+/− were mated with female abca1+/− mice. Genotyping of abca1+/− mice was carried out as described earlier.23 GFP mice were a generous gift from Dr David Gray at the University of Ottawa.26 C57 control mice were obtained from Charles River (Ontario). All animals are fed a regular chow diet and animal experimentation protocols were approved by the University of Ottawa Animal Care Committee.

Macrophage Cell Culture and 

3H-Cholesterol Labeling

To generate macrophages, bone marrow cells were harvested from femurs of abca1+/− or abca1+/− mice and differentiated by culturing in 15% L929 spent medium for 7 days. More than 97% of attached cells thus generated are macrophages, as indicated by cell expression of F4/80. The cells were then labeled by incubation with [3H]-cholesterol (5Ci/mL) preequilibrated with LDL or AcLDL (50 μg/mL) in DMEM containing 1% FBS for 24 hours. The labeled macrophages were washed and harvested by treatment with 10 mmol/L EDTA for 20 minutes at 37°C. The resuspended cells were washed twice with cold DMEM with 2 μg/mL BSA. The cells were then further incubated with 20 mL of 2 μg/mL BSA DMEM medium for an additional 1 hour. The final cell preparation was reconstituted in 300 μL for each mouse and injected either intraperitoneally or subfascially in the lumbar region. 24 hours later, blood, feces, gallbladders, and livers were harvested to measure radioactivity.

Measurement of Radioactivity and Determination of RCT

The distribution of [3H]-cholesterol between free cholesterol and cholesteryl ester was determined by thin-layer chromatography (TLC). Feces were collected over a 24-hour period. Before sacrifice, mice were fasted for 5 hours and anesthetized. Blood samples, liver, and gallbladder contents were collected. Radioactive counts in serum and gallbladder were measured directly by scintillation counting. HDL-associated [3H]-cholesterol and non-HDL-associated [3H]-cholesterol were measured after precipitation with phosphotungstic acid and magnesium. Livers were removed and weighed. A portion (1/5 to 1/10 of liver by weight) was excised, weighed, homogenized, and digested with 5 mL of 0.5 mol/L NaOH. Liver lipids were also extracted with isopropyl alcohol-hexane and the proportion of [3H]-cholesterol as free cholesterol and cholesteryl ester was determined by TLC. The [3H]-tracer detected in fecal bile acids was determined as previously described.2 The amount of [3H]-tracer was also expressed as a fraction of the injected dose.

Cellular Total Cholesterol and Free Cholesterol Analysis

Macrophages were labeled and loaded with LDL or AcLDL (50 μg/mL) without radioactive tracer for 24 hours as in the in vivo experiments. Cellular lipids were isolated isopropyl alcohol. The cellular levels of free and total cholesterol were analyzed according to the kit instruction (BioVision Research products). The fluorescence protocol was adopted and measured at Ex/Em=535/590 nmol/L in a microplate reader, because the sensitivity of this assay was <0.2 μg/well cholesterol.

Statistics

Results are expressed as mean±SD. One-way student t test was used. Sigma Plot, Excel, and Prism software were used.

Results

Characterization of Cholesterol-Labeled and -Loaded Macrophages

Cellular levels of total and free cholesterol were determined in macrophages labeled with AcLDL or LDL under the conditions used for in vivo RCT. There was no significant effect of the abca1 genotype on the levels of cholesterol in these cells, but as expected AcLDL loaded cells accumulated more cholesterol than LDL loaded cells as shown for a representative experiment (supplemental Table I, available online at http://atvb.ahajournals.org). The distribution of free and total [3H]-cholesterol was also measured after labeling with either [3H]-cholesterol-acLDL or -LDL (supplemental Figure I). Total cholesterol labeling did not differ between genotypes (supplemental Figure IA and IB). The relative proportion of esterified cholesterol derived from AcLDL also did not differ between genotypes (supplemental Figure ID), but the percentage of cholesteryl esters formed from LDL derived cholesterol was consistently higher in abca1+/− macrophages compared with heterozygote and wild-type (supplemental Figure IC).

In each of our in vivo RCT studies, the total [3H]-cholesterol tracer amounts in wild-type and ABCA1-inactivated macrophages that were injected in wild-type mice were similar. The means for all LDL labeled abca1+/− and abca1+/− macrophages injected in wild-type were 653 000 and 712 000 cpm/mouse, respectively. For AcLDL labeled-macrophages, there was again no significant difference between injected abca1+/− and abca1+/− macrophages (with means of 1 608 000 versus 2 056 000 cpm/mouse, respectively).

ABCA1 Deficiency in Macrophages Labeled With AcLDL-Derived Cholesterol Decreases RCT

To evaluate whether macrophage abca1 expression affects in vivo RCT, bone marrow–derived macrophages from abca1+/−, abca1+/−, or abca1+/− mice were loaded and labeled with [3H]-cholesterol-acLDL-labeled, injected intraperitoneally into their parental wild-type mice (C57), and transport of labeled cholesterol to blood, liver, gallbladder, and feces was measured. Preliminary experiments showed a clear gene dose effect with RCT decreasing with decreasing abca1 expression in macrophages (data not shown). The interanimal variation of the RCT method was also found to be large with a number of outlier data points (>2SD). To address this problem we compared alternate sites for injection of macrophages, namely intraperitoneal and subfascially in the lumbar (perirenal) region. The later presented a significant improvement in reproducibility in parallel experiments, where the standard deviations for transfer of radioactivity to serum were 58% and 25% for intraperitoneal and lumbar injections, respectively. All RCT results presented were first obtained by intraperitoneal injection and subsequently repro-
duced by lumbar injection. The 2 methods yielded similar RCT values and only differed in variability. These studies required a large number of animals, obtained by breeding homozygous null males with heterozygous females, which generated large numbers of homozygotes and heterozygotes that were used in all subsequent RCT studies. To ascertain that the labeled macrophages did not migrate to the liver, fluorescent GFP-macrophages were injected intraperitoneally and in the lumbar region. We did not observe any measurable fluorescence in either liver homogenates or histology sections, nor did we observe any measurable fluorescence in blood.

Serum radioactivity in wild-type mice measured at 24 hours was 33% lower after injection of abca1−/− macrophages as compared with abca1+/+ macrophages (Figure 1A). The results shown are the average of 2 experiments with 10 animals in each condition (P<0.01 for each experiment). The tracer distribution between HDL and non-HDL fractions was not altered (70% in HDL). Serum radioactivity was initially measured in blood samples taken at 24 and 48 hours but peaked at around 24 hours, in agreement with earlier results.2,3

Consistently, RCT from AcLDL-labeled and loaded macrophages showed that the liver radioactivity of animal injected with abca1−/− macrophage was 25% lower than that of mice injected with abca1+/+ macrophage (Figure 1B). Similarly, the excretion of 3H-tracer into bile and feces was significantly lower (31 and 35%, respectively) after injection of abca1−/− macrophages as compared with abca1+/+ cells (Figure 1C and 1D). These results, which are the mean of 5 separate experiments (each with 5 to 10 animals per condition), demonstrated that deficiency of macrophage abca1 significantly reduces the RCT from macrophages. Comparison of RCT values obtained with AcLDL-labeled abca1+/+ or −/− macrophages injected in wild-type mice indicated that the transfer of radioactivity to liver and bile was reduced by 51%, and 37%, respectively. These results are compatible with the extrapolation of RCT comparison of abca1−/− and abca1+/+ macrophages to that of abca1−/− and abca1+/+ macrophages, all injected in wild-type mice, representing a decrease of about 50%.

**ABCA1 Deficiency in Macrophages Labeled With LDL-Derived Cholesterol Decreases RCT**

We have recently demonstrated that 3H-cholesterol derived from LDL, as compared with AcLDL, delivers significantly more radioactivity to the recycling compartment, which effluxes preferentially to HDL.27 Macrophages incubated with high concentrations of native LDL can become foam cells and contribute to the development of atherosclerotic lesion.28–30 Thus we also analyzed RCT from macrophages loaded and labeled with native LDL. Our previous in vitro experiments had shown that cholesterol efflux from LDL labeled abca1−/− macrophages to apoA-I and HDL were also reduced.27 Here, we evaluated whether this reduced efflux was reflected in reduced RCT in vivo. Macrophages from abca1−/− or abca1+/+ mice were labeled with 3H-cholesterol delivered by LDL and injected into wild-type mice (Figure 2). Transfer of radioactivity into blood decreased by 62% and label was equally distributed between HDL and non-HDL lipoprotein fractions (Figure 2A). Transfer of radioactivity into liver, bile, and feces was reduced by 39, 32, and 48%, respectively, when comparing abca1−/− and abca1+/+ macrophages (Figure 2B, 2C, and 2D).

**Murine abca1 Inactivation and its Consequent HDL Deficiency Contribute to Decreased RCT**

From Cholesterol-Labeled Control Macrophages

Previous studies of RCT from various tissues showed that hepatobiliary cholesterol secretion and fecal sterol excretion were independent of ABCA1 deficiency,31 whereas others demonstrated a significant decrease in cholesterol absorption...
and an increase in whole body cholesterol, suggesting a markedly altered cholesterol metabolism. In these studies, reverse cholesterol transport included cholesterol derived from all tissues or tissue fluids and not only macrophages. It is now apparent that cholesterol transport from macrophage foam cells might not contribute significantly to whole organism RCT, and that cholesterol returned to the liver from other tissues might not be solely dependent on HDL and apoA-I. Here we evaluated whether HDL plasma level contributed to RCT from macrophage foam cells in addition to abca1 deficiency by taking advantage of the HDL-deficient phenotype in abca1-null mice. We labeled macrophages from wild-type mice with 3H-cholesterol-acLDL and injected them into abca1+/− or abca1−/− mice.

As expected, plasma radioactivity derived from AcLDL cholesterol–loaded control macrophages injected in abca1+/− mice was profoundly decreased by 85% compared with injection into abca1+/− mice (Figure 3A), reflecting the impairment of RCT attributable to the additive effects of hepatic abca1 deficiency and deficiency of circulating HDL cholesterol pool compared with heterozygous and wild-type mice. Interestingly, radioactivity not only decreased in the HDL cholesterol pool (about 17-fold), but also in non-HDL pool (about 4-fold; data not shown), indicating efflux and transfer to apoB or apoE-containing lipoproteins. In addition, bile radioactivity measured at the 24-hour period in abca1+/− mice was significantly reduced by 35% compared with abca1+/− mice. Furthermore, the 3H-tracer in liver of abca1+/− mice was significantly lower than in abca1+/− mice by 42% (Figure 3B and 3C). These results clearly showed that HDL and apoA-I play an important role in the RCT from macrophage foam cells.

**Discussion**

Several recent studies have analyzed the contribution of the sequential steps to organism-wide RCT. Alam et al. upregulated individual components and pathways involved in RCT, including apoA-I synthesis, circulating HDL concentrations, apoA-I–phospholipid complexes, SR-BI expression, LCAT activity, 7α-hydroxylase activity. However, these investigators could not identify a specific step that was limiting to the flux of cholesterol through the entire pathway, leading to impaired excretion of fecal sterols and bile acids. This study highlighted the complexity of organism-wide RCT, which is compounded by the major export contribution made by liver to the HDL cholesterol pool. Hepatic ABCA1 contributes to the initial lipiddation of newly synthesized apoA-I and to the circulating pool of HDL. Therefore, hepatic ABCA1 contributes both to export of cholesterol to the HDL pool and to the maintenance of HDL particles in the circulation allowing interaction with cells in other organs, particularly in the vascular system. Liver specific expression of SR-BI is independent of ABCA1 activity. Other recent studies of organism-wide RCT have had unexpected results. Hepatobiliary cholesterol transport is not impaired in abca1-null mice despite very low HDL concentrations, nor is the hepatic expression of genes controlling cholesteryl metabolism and biliary cholesterol output. Other studies showed that LXR regulation of hepatobiliary excretion was independent of ABCA1 activity.

These studies would appear to cast a doubt on the importance of RCT as a major transport pathway that delivers cholesterol for excretion. However, the hepatic turnover of cholesterol is many-fold greater than the amount of cholesterol shuttling between the liver and vascular cells and macrophage foam cells, which control lesion progression or regression. The method of macrophage-specific RCT of Zhang and colleagues circumvents the complexity of organism-wide RCT and focuses on the flux of cholesterol transport.
from the cell type of greatest interest to atherosclerosis. Using this method, we have shown that inactivation of abca1 in murine macrophages labeled with $^3$H-cholesterol-LDL markedly impairs RCT, with 25%, 31%, and 35% decreases in transfer to liver, bile, and feces, respectively (Figure 2). Because we compared abca1$^{-/-}$ and abca1$^{+/+}$ macrophages, extrapolation to the net decrease between abca1$^{-/-}$ and wild-type macrophages would be expected to exceed 50% as demonstrated in specific experiments.

Labeling and loading macrophages with cholesterol pre-equilibrated with AcLDL effectively delivers 10%, 20%, and 70% of label at the plasma membrane, recycling compartment, and late endosome, respectively, thus essentially enriching the recycling compartment by comparison to AcLDL. In abca1$^{-/-}$ macrophages, this results in higher efflux to HDL and little to no residual efflux to apoA-I. Here we showed that RCT from abca1$^{-/-}$ macrophages labeled with $^3$H-cholesterol-LDL decreases significantly by 25%, 31%, and 35% for transfer to liver, bile, and feces, respectively, compared with abca1$^{+/+}$ cells. This demonstrates that macrophage ABCA1 is a major contributor to cholesterol efflux and in vivo RCT, irrespective of the nature of cholesterol loading with native or modified LDL as indicated in our cellular cholesterol analysis (supplemental Figure I), in agreement with its role in promoting regression of lesion.

The previous comparison of RCT from wild-type macrophages labeled with either AcLDL or LDL and injected in wild-type mice showed that the transfer of AcLDL-derived cholesterol back to the liver and to bile or feces was 50% and 35% lower than that of LDL-derived cholesterol, an unexpected result, which most likely reflects the greater efficiency of HDL as a mediator of efflux in vivo. This is also in keeping with the marked decrease observed here in the transfer of cholesterol to serum when comparing wild-type macrophages injected intraperitoneally into abca1$^{-/-}$ mice (Figure 3A). This in turn explains why the residual HDL-mediated RCT of AcLDL-cholesterol from abca1$^{-/-}$ macrophages to bile and feces remained in the range of 65% to 69% compared with abca1$^{+/+}$ cells (Figure 1).

In conclusion, macrophage ABCA1 expression significantly decreases atherosclerotic lesions in mice, and here we show for the first time that inactivation of macrophage ABCA1 significantly decreases RCT from macrophages to liver, bile, and feces by about 50%, therefore demonstrating that ABCA1-dependent RCT is a functional mechanism that correlates with the antiatherogenic property of this transporter. In the absence of ABCA1 activity, however, an important residual RCT from macrophages remains evident, which demonstrates that other transporters that efflux to HDL are significant contributors to in vivo RCT.

Acknowledgments
We thank Drs Ruth McPherson, Ross Milne, and Stewart Whitman for critical reading of the manuscript.

Sources of Funding
This work was supported in part by grants from CIHR (#44359) and Heart and Stroke Foundation of Ontario (T5911) to Y.L.M.

Disclosures
None.

References


11. Eschugan AM, Oram JF. ABCA1 and ABCG1 or ABCG4 act sequentially to remove cellular cholesterol and generate cholesterol-rich HDL. *J Lipid Res*. 2006;47:2433–2443.


In Vivo Reverse Cholesterol Transport From Macrophages Lacking ABCA1 Expression Is Impaired
Ming-Dong Wang, Vivian Franklin and Yves L. Marcel

Arterioscler Thromb Vasc Biol. 2007;27:1837-1842; originally published online May 31, 2007;
doi: 10.1161/ATVBAHA.107.146068
Arteriosclerosis, Thrombosis, and Vascular Biology is published by the American Heart Association, 7272
Greenville Avenue, Dallas, TX 75231
Copyright © 2007 American Heart Association, Inc. All rights reserved.
Print ISSN: 1079-5642. Online ISSN: 1524-4636

The online version of this article, along with updated information and services, is located on the
World Wide Web at:
http://atvb.ahajournals.org/content/27/8/1837

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in Arteriosclerosis, Thrombosis, and Vascular Biology can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial Office. Once the online version of the published article for which permission is being requested is located, click Request Permissions in the middle column of the Web page under Services. Further information about this process is available in the Permissions and Rights Question and Answer document.

Reprints: Information about reprints can be found online at:
http://www.lww.com/reprints

Subscriptions: Information about subscribing to Arteriosclerosis, Thrombosis, and Vascular Biology is online at:
http://atvb.ahajournals.org//subscriptions/