Novel In Vivo Method for Measuring Cholesterol Mass Flux in Peripheral Macrophages

Ginny L. Weibel, Sara Hayes, Aisha Wilson, Michael C. Phillips, Jeffrey Billheimer, Daniel J. Rader, George H. Rothblat

Objective—Reverse cholesterol transport is the process by which excess cholesterol is removed from peripheral tissue by HDL and delivered to the liver for excretion. Presently, methods of measuring in vivo reverse cholesterol transport do so by monitoring the appearance in the feces of labeled cholesterol that originated from peripheral macrophage foam cells. These methods do not account for changes in macrophage cholesterol mass. We have developed an in vivo assay to measure cholesterol mass changes in atherosclerotic foam cells.

Methods and Results—Macrophages are entrapped in semipermeable (pore size 0.2 μm) hollow fibers and surgically implanted into the peritoneum of recipient mice. The fibers are removed from the peritoneum 24 hours after implantation. This method allows the complete recovery of the macrophages for quantification of changes in cholesterol mass and cellular protein. In wild-type mice we measured a significant reduction in total cell cholesterol (TC) when hollow fibers containing cholesterol-enriched macrophage cells were implanted (TC before implantation = 105 ± 18 μg/mg cell protein, TC 24 hours after implantation = 60 ± 16 μg/mg protein). Additionally, there was an increase in cholesterol content when hollow fibers containing cholesterol-normal macrophages were implanted in an atherogenic mouse model (LDLr/apobec dko) compared to a wild-type mouse (initial TC content = 57 ± 24 μg/mg protein, TC 24 hours after implantation: wild-type mice = 52 ± 10 μg/mg protein; LDLr/apobec dko mice = 118 ± 27 μg/mg protein).

Conclusion—This assay can quantify in vivo both cholesterol mass accumulation and reduction, in macrophages. This method permits quantitative analysis of the progression and regression of foam cells. (Arterioscler Thromb Vasc Biol. 2011;31:2865-2871.)

Key Words: lipids ■ lipoproteins ■ macrophages ■ reverse cholesterol transport ■ atherosclerosis

The progression of atherosclerosis is linked to the accumulation of cholesterol in foam cells of macrophage and smooth muscle origin. These foam cells are the hallmark of an atherosclerotic plaque. Macrophages and some smooth muscle cells store excess unesterified cholesterol (free cholesterol, FC) in the form of cholesteryl ester (CE) in cytoplasmic lipid droplets. The hydrolysis of droplet-CE in peripheral foam cells is critical to RCT because it represents the first step in cellular cholesterol clearance, as only FC is effluxed from cells. Because peripheral cells are not capable of catabolizing cholesterol, a mechanism is required to prevent this overaccumulation of cholesterol in cells such as macrophages in the walls of blood vessels. RCT was first described by Glomset in 1968 as the process by which peripheral cholesterol is transported to the liver, excreted in the bile and eliminated from the body in the feces.¹ The RCT pathway has gained significant attention as a target for therapeutic intervention. In fact, promotion of RCT by elevation of plasma HDL levels is thought to be the basis of epidemiological evidence that serum HDL levels are inversely proportional to the risk of coronary artery disease.²,³ Presently, methods of measuring in vivo RCT do not account for changes at the level of the foam cell.

Until recently, measuring the egress of cholesterol from the periphery to the feces has proven to be difficult. Our laboratory previously developed an in vivo assay of RCT that traces movement of radio-labeled cholesterol originating from cholesterol-enriched macrophage cells in the peritoneal cavity into the blood stream and subsequent fecal elimination.⁴ This protocol uses [³H]cholesterol-enriched macrophages (enriched and labeled ex vivo), which are injected into the peritoneum of recipient mice. Movement of [³H]cholesterol from the macrophages into the plasma compartment and elimination in the feces is measured 48 hours after injection. Distribution of label in liver and bile is also determined. Our initial studies using this method demonstrated that overexpression of apolipoprotein A-I promotes RCT.⁴ Since this study, we and others have used this method to determine the
impact of a wide variety of factors (eg, cholesteryl ester transfer protein, ATP-binding cassette transporter 1 [ABCA1], scavenger receptor class B type I, phospholipid transfer protein) on RCT. Although this method has proven to be a sensitive indicator of cholesterol movement in vivo, some concerns about this method have been raised. Among these are the following: (1) Is radiolabeled cholesterol an accurate tracer of cholesterol mass movement since measurement of radiola-
beled cholesterol as an indicator of mass movement does not account for any bidirectional flux or exchange of cholesterol in the peripheral macrophages. (2) Does the radiolabeled cholesterol detected in the serum represent intact macro-
phages that have migrated out of the peritoneal cavity and therefore does not represent actual flux of cholesterol? (3) Is there an actual reduction in macrophage foam cell CE mass since the appearance of labeled cholesterol in the plasma gives no indication of reduction in cholesteryl-free and esterified mass.

We have addressed these concerns and developed a novel method using hollow fibers so that the peritoneal macrophages can be recovered at the end of the RCT experiment, allowing us to quantitate cholesterol mass and cellular protein to assess changes in these parameters that occur during the experiment. With this novel method we can measure how manipulating the RCT pathway impacts on cholesterol status of peripheral macrophages, a primary source of lipid in atherosclerotic plaques.

Methods

Materials

Bovine serum albumin (essentially fatty acid-free), heat-inactivated fetal bovine serum, gentamicin, and unesterified cholesteryl ester (FC) were purchased from Sigma-Aldrich (St. Louis, MO). Organic solvents were obtained from Fisher Scientific (Pittsburg, PA) and [1,2-3H]cholesterol (5 CI/mmol) was obtained from New England Nuclear (Waltham, MA). Tissue-culture flasks and plates were purchased from Gibco-Invitrogen (Carlsbad, CA). Human LDL (1.019 < d < 1.063 g/mL) was isolated by sequential ultracentrifugation. With this novel method we can measure how manipulating the RCT pathway impacts on cholesterol status of peripheral macrophages, a primary source of lipid in atherosclerotic plaques.

Suspension Cell Cultures

Mouse peritoneal macrophages (MPMs) were isolated from male C57BL/6 mice (Jackson Laboratory, Bar Harbor, ME) 5 days after intraperitoneal injection with 2000 mg/kg 10% thioglycolate (Difco Laboratories, Detroit, MI) as previously described. Cells were washed twice in phosphate buffered saline (PBS) and suspended in RPMI containing 5% FCS. [3H]-cholesterol, 25 μg/mL acLDL, and 1% fetal bovine serum at 0.4 million cells/mL to label and choles-
teryl-enrich the macrophages. The cells were added to sterile Teflon flasks and put on a rotating platform to prevent adherence. Typically the macrophages contained 100 to 250 μg total cholesterol/mg cell protein after enrichment. Variations in batches of acLDL and cell density accounted for the range in cholesterol-enrichment. After the enrichment period (24 hours), the cells were pelleted and washed twice in minimum essential media (MEM) media and resuspended in RPMI containing 0.2% bovine serum albumin for 2 hours to allow the cellular pools of cholesterol to equilibrate before use in an experiment. All media were supplemented with 50 μg/mL gentamicin.

Hollow Fiber Preparation

Polyethersulfone (PES) hollow fibers (Microkros, Spectrum Laboratories, Rancho Dominguez, CA) with a 0.2-μm pore size and a 1-mm inner diameter were used. Hollow fibers were purchased as part of a filtration module. The entire module was autoclaved to sterilize it, after which the fibers were removed from the housing. Before being filled with MPM suspensions, the fibers were soaked in 100% ethanol for 30 minutes and flushed with MEM HEPES. Macrophages were cholesterol-enriched and labeled as described above. The macrophages were pelleted by low-speed centrifugation (2000 rpm, 10 minutes) and resuspended in RPMI media at a cell concentration of 7 x 10^6 cells per 100 μL. A 100 μL aliquot of the cell suspension was added to a 2-cm length of a fiber that had one end heat-sealed using a smooth jawed hose clamp that was heated using a heating block set at 180°C. Cells were inserted into the fiber using a 1-mL syringe equipped with a 21-g needle. The open end of the fiber was then heat-sealed as described above and the fiber was then incubated for 5 minutes in RPMI media with gentle shaking to evenly disperse the cells within the fiber.

Cellular Toxicity

Cellular toxicity was determined by release of lactate dehydrogenase (Roche kit, Basel, Switzerland) into the medium. Cell viability was also determined by trypan blue (Invitrogen, Carlsbad, CA) exclusion (as per manufacturer’s instructions).

In Vitro Cholesterol Efflux

Hollow fibers containing [3H]-cholesterol–enriched macrophages (prepared as described above) were placed in 6-well tissue culture plates (1 fiber per well). [3H]-cholesterol enriched MPM cells in suspension were seeded in Teflon flasks. Media containing either mouse serum (to act as an extracellular cholesterol-acceptor) or 0.2% bovine serum albumin was added to each well/flask for 24 hours. To determine cholesterol efflux, media were sampled at 24 hours, filtered, and counted by liquid scintillation counting to determine [3H] released. [3H]-sterols in the media were isolated by sequential ultracentrifugation, diazylated against 0.15 mol/L NaCl, and sterilized by filtration. LDL was converted to acetylated LDL (acLDL) by treatment with acetic anhydride. 11

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Animals

Wild-type C57BL/6 mice were obtained from the Jackson Labora-
tory (Bar Harbor, ME). LDLR/apobec double knockout mice were bred in-house (originally from Genentech Inc, San Francisco, CA). The weight of the mice used in the experiments ranged from 22 to 37 g. Mice were fed a standard chow diet ad libitum before and during the study. For plasma lipid analyses, animals were fasted for 4 hours and then bled from the retro-orbital plexus. All animals were housed according to guidelines of the Institutional Animal Care and Usage Committee of the University of Pennsylvania. All protocols were considered and approved by the Institutional Animal Care and Usage Committee.

In Vivo Assays

Hollow Fiber Method

On the day of the surgery, animals were divided into groups (N = 3). Mice were anesthetized with a mixture of 100 mg/kg ketamine and 10 mg/kg xylazine administered intraperitoneally. Mice were shaved at the lower dorsal (site of incision) and the site was wiped with an alcohol swab. An incision was made perpendicular to the long axis of the mouse to expose the abdominal musculature and this was followed by a second small incision to access the peritoneal cavity. Two hollow fibers (prepared as described above), each 2 cm in length, were fed through the small incision (approximately 0.5 cm) into the peritoneal cavity. After insertion of the fiber, the musculo-
peritoneal layer was closed with absorbable suture. The skin incision
was then closed with suturing, and 1 mg/kg (0.1%) buprenorphine or 5 mg/kg of meloxicam was administered subcutaneously immediately before closure and 8 to 12 hours post-surgery to provide an analgesic effect. Blood was collected at 24 hours by retro-orbital bleed and plasmas were used for liquid scintillation counting. The animals were euthanized 24 hours after implantation and the fibers were removed and equilibrated in 10 mL PBS at 37°C for 10 minutes. The fibers were washed twice in PBS containing 0.2 mg/mL EDTA for 5 minutes and once in trypsin for 10 minutes. Fibers were stored in PBS at room temperature until analyzed.

**Injection Method**

MPM were cholesterol-enriched and labeled by incubation with [3H]cholesterol (1 µCi/mL) and acDL (100 µg protein/mL) and were injected (typically 4.5×10^6 cells containing 7.5×10^6 cpm in 0.5 mL DMEM) intraperitoneally as described previously.4 After the RCT period, tissues were collected and analyzed for total radioactivity and feces were collected continuously from 0 to 24 hours and stored at 4°C before extraction and analysis as previously described.4

**Protein and Cholesterol Determination**

After removal of the fibers from the animals and washing as described above, both ends of the fiber was cut open and the cells were flushed out using 1 mL PBS. The cells were pelleted by low-speed centrifugation. Lipids were extracted from the cell pellets with isopropanol containing cholesteryl methyl ether as an internal GLC standard. Total and free cholesterol was quantified by gas-liquid chromatography.13 After extracting the lipids from the cells, protein was solubilized by incubating the cell pellet in an SDS buffer. Protein was measured by the method of Markwell et al.14

**Data Analysis**

Data are from representative experiments and are expressed as mean±standard deviation. Statistical test for significance was performed using an unpaired t test with a 95% confidence interval.

**Results**

**In Vitro Validation of Cholesterol Efflux From Cells Entrapped in Hollow Fibers**

PES fibers were purchased from Spectrum Laboratories (Rancho Dominguez, CA). PES fibers were chosen because they are made of an inert material, they are flexible, and they have a pore size of 0.2 µm. This pore size allows for the retention of cells while permitting the flux of lipoproteins through the fibers. We first determined, in an in vitro experiment, if entrapment of the macrophage cells within the fibers would result in cellular toxicity (measured by trypan blue exclusion and lactate dehydrogenase release). For this, we placed MPMs into a fiber as described in Methods. Three sections of the fibers were incubated in media containing 10% fetal bovine serum. Parallel incubations of MPM cells in suspension were used as controls. After 24 hours lactate dehydrogenase in the media was determined in the hollow fiber wells and in the suspension cultures. There was no statistical difference in lactate dehydrogenase release between the cells in the hollow fibers and the cells grown in suspension (cells in hollow fibers: 9.5±0.2 absorbance units/mg cell protein; cells in suspension: 8.7±1.6 absorbance units/mg cell protein, N=3). Additionally the fibers were removed from the incubation media after 24 hours and both ends were cut open. The cells were flushed out with PBS. The cells were then subject to viability analysis by trypan blue exclusion. Cells were viewed using light microscopy. Random fields and a total of 1,000 cells from each fiber or 1,000 cells from a suspension culture were counted. We determined that viability was greater than 96% after the 24 hours incubation in the hollow fibers (cells in suspension viability 96.6±2.2%; cells flushed from hollow fibers 96.7±0.5%). To determine if the fibers would permit the free exchange of serum components (eg, lipoproteins) while retaining the macrophage cells, we conducted several in vitro experiments. Cholesterol mass change in cholesterol-enriched MPM contained in a 5-cm length of hollow fiber and submerged in 10% mouse serum was determined at 24 hours. The 24-hour time point was chosen to allow for sufficient hydrolysis of stored CE as only unesterified cholesterol (FC) can be exported from cells. The data in Figure 1A indicate that there was no significant difference in efflux of radiolabeled cholesterol from cells in suspension and cells entrapped within the porous hollow fibers. Importantly, we measured a significant difference in reduction of FC (P=0.04) and CE (P=0.02) across the mass before the incubation (t=0) when the macrophage-containing fibers were incubated in the presence of an extracellular cholesterol acceptor (10% mouse serum, Figure 1B) but no significant difference when there was no extracellular acceptor present (MEM, Figure 1B).

![Figure 1](http://atvb.ahajournals.org/)

**Figure 1.** In vitro validation of the hollow fiber method. Mouse peritoneal macrophages were cholesterol-enriched and labeled by incubation with acDL and [3H]cholesterol and then injected into hollow fibers or seeded in Teflon flasks for growth in suspension. The fibers and suspension cells were incubated at 37°C for 24 hours in tissue culture media containing 10% mouse serum. For efflux measurements (A) media from the wells containing the fibers and an aliquot of the suspension cell incubation was sampled and filtered through a 0.4 µm filter. Total [3H] was determined and percent efflux was calculated by comparing [3H] in the tissue culture medium to total radioactivity in the cells before the efflux period. Cells were then flushed out of the fibers and assayed for cholesterol mass content (GLC), total radioactivity (LSC), and protein. Free- and esterified cholesterol mass was determined in cells at the beginning of the incubation period (t=0) and after being incubated for 24 hours in media alone (MEM) or 10% mouse serum (MS, B). N=3 determinations from a single experiment. Experiment was repeated twice. *Significantly different from t=0 group.
In Vivo Hollow Fiber Cellular Cholesterol Flux Assay

Because we determined that the PES fiber was suitable for retention of cells and allows for the free exchange of serum lipoproteins, we next optimized the assay for use in vivo. The mice receiving the fibers ranged in size from 22 to 37 g. The surgery for implantation was designed to allow for minimal discomfort and quick recovery. The incisions made were just large enough to feed the fibers into the peritoneal cavity (approximately 0.5 cm). The mice recovered within a few hours and were able to move and eat freely. We determined that the maximal length of the fiber was 2 cm. We were not able to insert longer segments into the cavity smoothly and they tended to kink. Originally we attempted to insert 3 fibers per mouse but found that 3 fibers caused kinking and damage to the fibers and some bleeding in the cavity. We found that the peritoneal cavity of the mice sustained 2 fibers without any kinking or bleeding. We typically removed the fibers after 24 hours, but we also extended some of our preliminary experiments to 48 hours with no ill effects to the animals.

After removal from the peritoneum, the fibers are equilibrated in 10 mL of PBS at 37°C to dilute out any peritoneal fluid trapped in the fiber. Additionally, in our initial attempts at inserting the fibers into the peritoneal cavity and recovering them after 24 hours, we found that the fibers accumulated resident cells on the exterior of the fibers. EDTA and trypsin washes (see Methods) of the fibers after removal from the peritoneum were added to remove any cells that adhered to the fiber’s surface.

Hollow fibers containing [3H]-cholesterol–enriched MPM were implanted into the peritoneal cavity of wild-type mice as described in Methods and shown in Figure 2. An RCT experiment using [3H]-cholesterol-enriched MPM injected directly (as described in Methods) into the peritoneal cavity was run in parallel (in separate mice) with the hollow fiber method for comparison of radioactivity in the serum of the recipient animals. Radioactivity appearing in the serum (24 hours) of the animals was not significantly different between the mice receiving the macrophages in a hollow fiber versus mice receiving macrophages directly injected into the peritoneum (1.5±0.9% n=8 hollow fiber, 1.75±0.5% n=6 injection, reported as % of radioactivity originally introduced to the peritoneal cavity, P=0.6) indicating that the radiolabeled tracer cholesterol distributed similarly in both experimental
systems. The fibers were removed 24 hours after implantation and the MPM within the fibers were flushed out and collected as described in Methods. The resultant cells were analyzed for protein and cholesterol mass. There were significant reductions in total cholesterol, FC, and CE in the cells that were implanted in hollow fibers compared to the cholesterol content of the cells before the RCT period (Table 1). We next compared loss of cholesterol mass and loss of [3H]cholesterol from the fibers after 24 hours in the peritoneal cavity. There was a greater reduction in the tracer [3H]cholesterol compared to the reduction of cholesterol mass measured (Figure 3A). Additionally, the specific activity of the [3H]cholesterol in the macrophages that were implanted in the hollow fibers decreased when compared to the specific activity of the cellular cholesterol before implantation (t=0, Figure 3B). This data suggests that cellular cholesterol efflux as well as unlabeled cholesterol influx (evident from the decrease in cellular cholesterol specific activity) are important in determining total RCT.

The Effect of ABCA1 Expression on Cholesterol Mass Movement From Peripheral Cells

To further validate that the hollow fiber method can measure incremental changes in cholesterol mass movement from peripheral macrophages, we tested the method in ABCA1 knock-out mice. In these experiments, fiber macrophages in wild-type mice lost significant total- and esterified-cholesterol mass (42% loss in total cholesterol and 46% loss in CE) compared to the cholesterol mass in the cells before the implantation (T=0, Table 2). In contrast, the cholesterol levels in the fiber macrophages in the ABCA1 knockout mice did not change. Importantly, this system demonstrated that macrophage cholesterol mass differences can be detected in different mouse models.

**Cholesterol Deposition in Macrophages Within Hollow Fibers**

The experiments presented above clearly demonstrate that the hollow fiber RCT method can be used to quantitate the loss of cholesterol mass from cholesterol-enriched macrophages placed in the peritoneum of mice. We next determined if the hollow fiber assay could be used to study the accumulation of cellular cholesterol mass and the conversion of cholesterol-normal macrophages into cholesterol-enriched cells. Our experimental system used an atherogenic model, LDLR/apo-

<table>
<thead>
<tr>
<th>Time (h)</th>
<th>Total Cholesterol µg/mg Protein</th>
<th>Esterified Cholesterol µg/mg Protein</th>
<th>Free Cholesterol µg/mg Protein</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>105±18</td>
<td>51±18</td>
<td>53±13</td>
</tr>
<tr>
<td>24</td>
<td>60±16*</td>
<td>26±5*</td>
<td>34±12*</td>
</tr>
<tr>
<td></td>
<td>P=0.0001</td>
<td>P&lt;0.0001</td>
<td>P=0.003</td>
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</tbody>
</table>

Mouse peritoneal macrophages cells were cholesterol enriched by incubation with acLDL and entrapped within hollow fibers. The fibers were implanted into the peritoneal cavity of wild-type mice (C57BL/6) for 24 hours. Cholesterol mass was determined by GLC. N=10 determinations combined from 3 separate experiments. *Significantly different from time=0 value.

**Table 2. Effect of ABCA1 on Cholesterol Mass Movement**

<table>
<thead>
<tr>
<th>In Vivo</th>
<th>Total Cholesterol µg/mg Protein</th>
<th>Esterified Cholesterol µg/mg Protein</th>
<th>Free Cholesterol µg/mg Protein</th>
</tr>
</thead>
<tbody>
<tr>
<td>Time=0</td>
<td>218±30</td>
<td>149±26</td>
<td>69±24</td>
</tr>
<tr>
<td>Wild type</td>
<td>126±54*</td>
<td>80±34*</td>
<td>46±21</td>
</tr>
<tr>
<td>ABCA1 ko</td>
<td>197±36†</td>
<td>116±33†</td>
<td>81±13†</td>
</tr>
</tbody>
</table>

Mouse peritoneal macrophages cells were cholesterol enriched by incubation with acLDL and entrapped within hollow fibers. The fibers were then implanted into the peritoneal cavity of indicated mice for 24 hours. Cholesterol mass was determined by GLC. N=6 determinations from 2 separate experiments. *Significantly different from time=0. †Significantly different from wild type.
peritoneal cavity of recipient mice. Using this system, it is enriched in radio-labeled tracer cholesterol injected into the recently, RCT has been measured using macrophage cells content at the level of the macrophage. The method for evaluating in vivo cholesterol presented in this article represents a significant advance- approach because of the difficulty in recovering the cells from the macrophage and the distribution of labeled cholesterol- possible to determine the flux of radiolabeled cholesterol from the peritoneum. The method for evaluating in vivo cholesterol mass flux in peripheral macrophage cells through small incisions into the peritoneal cavity of recipient wild-type mice. After the fibers were recovered (24 hours) we measured a 43% decrease in total cholesterol mass with significant decreases in both FC and CE. There was an approximately 50% decrease in CE in 24 hours (Table 1) which is in agreement with the reported half-time of CE hydrolysis in MPM cells. This hollow fiber system makes it possible for the first time to quantify cellular cholesterol mass changes in vivo. The hollow fiber assay is sensitive enough to measure cholesterol mass changes in mouse models with differing capacities for cholesterol flux such as demonstrated by our experiments using ABCA1 knock-out and wild-type mice (Table 2). Serum levels of [3H]cholesterol measured at 24 hours were statistically equivalent to the levels observed in the RCT method where macrophages are injected directly into the peritoneum; this result indicates that the efflux of [3H]cholesterol from the peritoneal cavity was not hindered by the cells being entrapped in the fibers.

When comparing the loss of [3H]cholesterol from the cells entrapped in the fiber to the loss of cholesterol mass, we observed that there was a discrepancy between change in mass and isotope (Figure 3A). A greater reduction in [3H]cholesterol was measured compared to the reduction in cholesterol mass. Loss of [3H]cholesterol from specific cellular pools seems unlikely given that [3H]cholesterol readily equilibrates with nonlabeled cholesterol within cells and is an accurate tracer of total cellular cholesterol pool. We also determined that the specific activity of the cellular cholesterol was reduced approximately 40% at 24 hours. The reduction in specific activity could be due to either dilution of label by de novo synthesis, which is unlikely because synthesis would be down-regulated because the cells were already cholesterol-enriched, or due to influx of nonlabeled cholesterol from lipoproteins in the peritoneal fluid, which is a more plausible explanation. This observation demonstrates that while [3H]cholesterol tracer is a reliable tool for determining flux of cholesterol out of the periphery to HDL for excretion, it does not provide a complete description of cholesterol trafficking to and from peripheral macrophage foam cells.

Because our observations indicated that we are able to measure cholesterol mass influx in the cells entrapped within

### Table 3. Measurement and Validation of Cholesterol Accumulation in Mouse Peritoneal Macrophages

<table>
<thead>
<tr>
<th>In Vivo</th>
<th>Total Cholesterol µg/mg Protein</th>
<th>Esterified Cholesterol µg/mg Protein</th>
<th>Free Cholesterol µg/mg Protein</th>
</tr>
</thead>
<tbody>
<tr>
<td>Time=0</td>
<td>57±24</td>
<td>8±4</td>
<td>49±12</td>
</tr>
<tr>
<td>Wild type</td>
<td>52±10</td>
<td>14±3*</td>
<td>39±9</td>
</tr>
<tr>
<td>LDLR/apobec dko</td>
<td>118±27*</td>
<td>62±21*</td>
<td>56±13</td>
</tr>
</tbody>
</table>

Cholesterol-normal mouse peritoneal macrophage cells were entrapped within hollow fibers. The fibers were then implanted into the peritoneal cavity of indicated mice (in vivo) for 24 hours. Cholesterol mass was determined by GLC. N=6 determinations from two separate experiments.

*Significantly different from time=0.

LDLR/apobec dko, n=6 per group, P<0.0001) and develop atherosclerosis. The fibers were removed after 24 hours and the cells were flushed from the fibers and collected and assayed for protein and cholesterol mass. Total cellular cholesterol in the cells in the hollow fibers implanted in wild-type mice did not change when compared to the cholesterol mass at t=0. In contrast, total cellular cholesterol mass doubled in the MPM entrapped in fibers in the LDLR/apobec double knockout mice and this increase was entirely due to an 8-fold increase in CE content (Table 3).

### Discussion

**In Vivo Cholesterol Homeostasis**

Atherosclerosis is a chronic inflammatory disease that is associated with the accumulation of lipid-laden macrophage foam cells within the wall of an artery. The disease state progresses when there is inadequate removal of cholesterol from the macrophages by functional HDL for excretion by the liver in a process known as RCT. Therefore many efforts have been dedicated to understanding HDL function and factors that affect RCT. One of the first reported attempts to quantify RCT was published by Stein et al in 1999 wherein they injected a bolus of cationized LDL containing radio-labeled cholesterol into the flank of rodents and followed the disappearance of labeled-cholesterol from the site. Recently, RCT has been measured using macrophage cells enriched in radio-labeled tracer cholesterol injected into the peritoneal cavity of recipient mice. Using this system, it is possible to determine the flux of radiolabeled cholesterol from the macrophage and the distribution of labeled cholesterol in plasma, liver, and feces. However, it is not possible to monitor macrophage cholesterol homeostasis by this approach because of the difficulty in recovering the cells from the peritoneum. The method for evaluating in vivo cholesterol flux presented in this article represents a significant advancement in our ability to detect and quantify changes in lipid content at the level of the macrophage.

**Hollow Fiber Flux Assay**

In an attempt to measure cholesterol mass flux in peripheral cells we initially attempted to recover the macrophage cells from the peritoneal cavity after the cells were directly injected. We found that we could not quantitatively recover the injected cells as the cells stuck to the walls of the cavity and mixed with resident cells. Recovery by this method also does not account for any cells that may have migrated out of the peritoneum during the experimental period. We therefore developed a device to entrap the cells before inserting them into the peritoneum. Initially, we validated the use of the PES semipermeable fibers in vitro to determine if the membrane would allow for free exchange of lipoproteins and lipids across the barrier and yet retain the macrophage cells entrapped within the fibers in a viable state. Our results indicated that the cells within the semipermeable membrane had equivalent cholesterol efflux potentials when compared to cells grown in suspension indicating that extra-cellular cholesterol acceptors freely exchange across the membrane. Additionally, we were able to recover the cells from the fibers, determine that there was no decrease in viable cells, and quantify the cholesterol mass in the cells (Figure 1).

To test this system in vivo, we implanted two 2-cm segments of the fibers containing [3H]cholesterol-enriched macrophage cells through small incisions into the peritoneal cavity of recipient wild-type mice. After the fibers were recovered (24 hours) we measured a 43% decrease in total cholesterol mass with significant decreases in both FC and CE. There was an approximately 50% decrease in CE in 24 hours (Table 1) which is in agreement with the reported half-time of CE hydrolysis in MPM cells. This hollow fiber system makes it possible for the first time to quantify cellular cholesterol mass changes in vivo. The hollow fiber assay is sensitive enough to measure cholesterol mass changes in mouse models with differing capacities for cholesterol flux such as demonstrated by our experiments using ABCA1 knock-out and wild-type mice (Table 2). Serum levels of [3H]cholesterol measured at 24 hours were statistically equivalent to the levels observed in the RCT method where macrophages are injected directly into the peritoneum; this result indicates that the efflux of [3H]cholesterol from the peritoneal cavity was not hindered by the cells being entrapped in the fibers.

When comparing the loss of [3H]cholesterol from the cells entrapped in the fiber to the loss of cholesterol mass, we observed that there was a discrepancy between change in mass and isotope (Figure 3A). A greater reduction in [3H]cholesterol was measured compared to the reduction in cholesterol mass. Loss of [3H]cholesterol from specific cellular pools seems unlikely given that [3H]cholesterol readily equilibrates with nonlabeled cholesterol within cells and is an accurate tracer of total cellular cholesterol pool. We also determined that the specific activity of the cellular cholesterol was reduced approximately 40% at 24 hours. The reduction in specific activity could be due to either dilution of label by de novo synthesis, which is unlikely because synthesis would be down-regulated because the cells were already cholesterol-enriched, or due to influx of nonlabeled cholesterol from lipoproteins in the peritoneal fluid, which is a more plausible explanation. This observation demonstrates that while [3H]cholesterol tracer is a reliable tool for determining flux of cholesterol out of the periphery to HDL for excretion, it does not provide a complete description of cholesterol trafficking to and from peripheral macrophage foam cells.

Because our observations indicated that we are able to measure cholesterol mass influx in the cells entrapped within
the fibers using this RCT assay, we extended our studies to investigate conditions where cholesterol accumulation would occur in peripheral macrophages. These studies used an atherogenic mouse model, the LDLR/apobec double knockout (dko) mouse. When cholesterol-normal MPM were entrapped within fibers and the fibers were implanted in the peritoneal cavity of the LDLR/apobec dko mice, the MPM was converted into a cholesterol-enriched phenotype by doubling the total cholesterol mass in the cell and expanding the CE mass of the cell 8-fold. In comparison, the cholesterol-normal MPM that were implanted into wild-type mice had no increase in total cholesterol mass and a small expansion in the percent of the mass present as CE. This data clearly shows that cholesterol mass deposition in peripheral cells can be evaluated using this technique.

Methodological Advantages and Potential Uses

The hollow fiber method described in this manuscript allows for the complete recovery of the cells after the experimental period for analysis. This is the first in vivo assay to quantitatively assess changes in cholesterol mass in a defined cell population in the periphery. This method can evaluate how manipulations of the RCT pathway affect cholesterol status in peripheral macrophages. Thus this technique provides an accurate determination of in vivo cholesterol status as a measure of atherosclerosis progression and regression. This method, however, is not limited to use in atherosclerotic studies and could be modified to suit many experimental protocols or purposes. We developed the assay for use in mice, but it can easily be adapted for use in larger animals such as primates. The site of implantation could be subcutaneous rather than intraperitoneal. We saw no obvious deleterious effects of the fibers after 48 hours of implantation in the mice and we believe that long-term feeding studies may be feasible using this assay. Additionally, the assay is not limited to using wild-type macrophages as the cell-type entrapped in the fibers. Genetically altered cells or macrophages in different inflammatory states would be candidates for use in this system. This novel method has great potential to provide invaluable insight into the biological factors that promote atherosclerosis and aid in assessment and validation of new therapeutics such as those designed to promote plaque regression or stabilization.

Sources of Funding

This work was supported by NIH PPG grant HL22633.

Disclosures

Dr Rader and Dr Rothblat own equity in VascularStrategies, LLC.

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Novel In Vivo Method for Measuring Cholesterol Mass Flux in Peripheral Macrophages
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Arterioscler Thromb Vasc Biol. 2011;31:2865-2871; originally published online September 22, 2011;
doi: 10.1161/ATVBAHA.111.236406
Arteriosclerosis, Thrombosis, and Vascular Biology is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 1079-5642. Online ISSN: 1524-4636

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