Macrophage ABCG1 Deletion Disrupts Lipid Homeostasis in Alveolar Macrophages and Moderately Influences Atherosclerotic Lesion Development in LDL Receptor-Deficient Mice

Ruud Out, Menno Hoekstra, Reeni B. Hildebrand, Janine K. Kruit, Illiana Meurs, Zhaosha Li, Folkert Kuipers, Theo J.C. Van Berkel, Miranda Van Eck

Objective—ABCG1 has recently been identified as a facilitator of cellular cholesterol and phospholipid efflux to high-density lipoprotein (HDL). Its expression in macrophages is induced during cholesterol uptake in macrophages and by LXR. The role of macrophage ABCG1 in atherosclerotic lesion development is, however, still unknown.

Methods and Results—To assess the role of macrophage ABCG1 in atherosclerosis, we generated low-density lipoprotein (LDL) receptor knockout (LDLr−/−) mice that are selectively deficient in macrophage ABCG1 by using bone marrow transfer (ABCG1−/− → LDLr−/−). Peritoneal macrophages isolated from donor ABCG1−/− mice exhibited a 22% (P=0.0007) decrease in cholesterol efflux to HDL. To induce atherosclerosis, transplanted mice were fed a high-cholesterol diet containing 0.25% cholesterol and 15% fat for 6 and 12 weeks. Serum lipid levels and lipoprotein profiles did not differ significantly between ABCG1−/− → LDLr−/− mice and controls. In lungs of ABCG1−/− → LDLr−/− mice a striking accumulation of lipids was observed in macrophages localized to the subpleural region. After 6 weeks of high-cholesterol diet feeding the atherosclerotic lesion size was 49±12×103 μm2 for ABCG1+/+ → LDLr−/− mice versus 65±15×103 μm2 for ABCG1−/− → LDLr−/− mice and after 12 weeks of high-cholesterol diet feeding 124±17×103 μm2 for ABCG1+/+ → LDLr−/− mice versus 168±17×103 μm2 for ABCG1−/− → LDLr−/− mice. Atherosclerotic lesion size depended on both time and the macrophage ABCG1 genotype (P=0.038 by 2-way ANOVA, n=8), indicating a moderately 33% to 36% increase in lesion formation in the absence of macrophage ABCG1.

Conclusions—Macrophage ABCG1 deficiency does lead to heavy lipid accumulation in macrophages of the lung, and also a moderately significant effect on atherosclerotic lesion development was observed. (Arterioscler Thromb Vasc Biol. 2006;26:000-000.)

Key Words: ABCG1 ■ atherosclerosis ■ cholesterol ■ macrophage ■ transplantation

The transport of excess cholesterol from peripheral tissues back to the liver for catabolism and excretion in bile, called reverse cholesterol transport (RCT), plays an important protective role in the development of atherosclerosis. Several ATP-binding cassette (ABC) transporters, which constitute a large family of evolutionarily conserved transmembrane proteins that translocate a wide variety of substrates across cellular membranes, have been implicated in RCT. ABCA1 is involved in the first step of RCT: the efflux of cholesterol from peripheral tissue macrophages to lipid-free apolipoproteins.2–4 Macrophage ABCA1 expression is induced during cholesterol uptake5,6 and is regulated via LXR.7 In addition, depletion of ABCA1 in macrophages induces lesion development, while overexpression prevents the progression of atherosclerotic lesions.8–10

In addition to ABCA1, macrophages also express ABCG1, which is induced during cholesterol uptake in macrophages11,12 and is activated via LXR.13–15 In contrast to ABCA1, ABCG1 facilitates cellular cholesterol and phospholipid efflux from macrophages to mature high-density lipoprotein (HDL), but not to lipid-free apolipoproteins.11,16 ABCG1 plays a critical role in preventing cellular lipid accumulation within multiple tissues after administration of a high-fat, high-cholesterol diet16 and ABCG1 is expressed by macrophage-derived foam cells in the human atherosclerotic plaque.17 Macrophage ABCG1 expression has therefore been suggested to play a key role in RCT and the protection against atherosclerosis.

In this study, the potential role for macrophage ABCG1 in atherosclerosis was examined using the method of bone

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marrow transplantation. The results show that macrophage ABCG1 expression did substantially affect lipid homeostasis in macrophages of the lung, whereas macrophage ABCG1 expression also moderately influenced atherosclerotic lesion development.

Materials and Methods

Animals
ABCG1+/− mice, obtained from Deltagen Inc, San Carlos, California, were cross-bred to generate ABCG1+/− and ABCG1−/− mice. Genotyping for ABCG1 was performed according to the protocol from Deltagen. A multiplex reaction was performed to detect both wild-type and targeted alleles, whereas a second reaction was performed to detect only the endogenous wild-type allele. Homozygous low-density lipoprotein (LDL) receptor knockout (LDLr−/−) mice were obtained from The Jackson Laboratory (Bar Harbor, Me) as mating pairs and bred at the Gorlaeus Laboratory, Leiden, The Netherlands. Mice were housed in sterilized filter-top cages and given unlimited access to food and water. Mice were maintained on sterilized regular chow, containing 4.3% (w/w) fat with no added cholesterol (RM3; Special Diet Services, Witham, UK), or were fed a semisynthetic high-cholesterol diet containing 15% (w/w) fat and 0.25% (w/w) cholesterol (Diet W; Abbdiets, Woerden, The Netherlands). Drinking water was supplied with antibiotics (83 mg/L ciprofloxacin and 67 mg/L polymyxin B sulfate) and 6.5 g/L sucrose. Animal experiments were performed at the Gorlaeus Laboratories of the Leiden/Amsterdam Center for Drug Research in accordance with the National Laws. All experimental protocols were approved by the Ethics Committee for Animal Experiments of Leiden University.

Bone Marrow Transplantation
To induce bone marrow aplasia, male LDLr−/− mice were exposed to a single dose of 9 Gy (0.19 Gy/min, 200 kV, 4 mA) total body irradiation, using an Andrex Smart 225 Roentgen source (YXLON Int, Copenhagen, Denmark) with a 6-mm aluminum filter. Bone marrow was isolated by flushing the femurs and tibias from mice with phosphate-buffered saline. Single-cell suspensions were prepared by passing the cells through a 30-μm nylon gauze. Irradiated recipients received 0.5×106 bone marrow cells by intravenous injection into the tail vein. After a recovery of 8 weeks animals received a high-cholesterol diet for 6 or 12 weeks.

Assessment of Successful Bone Marrow Reconstitution
The hematologic chimerism of the LDLr−/− mice was determined in genomic DNA from bone marrow by polymerase chain reaction as described. Final polymerase chain reaction products were fractionated on a 2% agarose gel.

Macrophage Cholesterol Efflux Studies
Thioglycollate-elicited peritoneal macrophages were incubated with 0.5 μg/mL H-cholesterol in DMEM/0.2% bovine serum albumin for 24 hours at 37°C. To determine cholesterol loading, cells were washed 3 times with washing buffer (50 mMol/L Tris containing 0.9% NaCl, 1 mMol/L EDTA, and 5 mMol/L CaCl2, pH 7.4), lysed in 0.1 mol/L NaOH, and the radioactivity was determined by liquid scintillation counting. Cholesterol efflux was studied by incubation of the cells with DMEM/0.2% bovine serum albumin alone or supplemented with either 5 μg/mL apoAI (Calbiochem) or 50 μg/mL human HDL (density 1.63 to 1.21 g/mL), isolated according to Redgrave et al.4 Radioactivity in the medium was determined by liquid scintillation counting after 24 hours of incubation.

Lipid Analyses
After an overnight fasting-period, ~100 μL of blood was drawn from each individual mouse by tail bleeding. The concentration of triglycerides in serum was determined using an enzymatic colorimetric assay (Roche Diagnostics, Mannheim, Germany). Precipath (standardized serum; Roche, Germany) was used as internal standard. The concentrations of total cholesterol in serum were determined by enzymatic colorimetric assays with 0.025 U/mL cholesterol oxidase (Sigma), 0.065 U/mL peroxidase (Roche Diagnostics, Mannheim, Germany), and 15 μg/mL cholesteryl esterase (Roche Diagnostics) in reaction buffer (1.0 mol/L KPi buffer, pH = 7.7 containing 0.01 mol/L phenol, 1 mmol/L 4-amino-antipyrine, 1% polyoxyethylene-9-lauryl ether, and 7.5% methanol). Absorbance was read at 490 nm. The distribution of cholesterol over the different lipoproteins in serum was determined by fractionation of 30 μL of serum of each mouse using a Superox 6 column (3.2×300 mm, Smart-System; Pharmacia, Uppsala, Sweden). Total cholesterol content of the effluent was determined as described.

Histological Analysis of the Aortic Root
To analyze the development of atherosclerosis at the aortic root, transplanted LDLr−/− mice were euthanized after 6 and 12 weeks of feeding the high-cholesterol diet. The arterial tree was perfused in situ with phosphate-buffered saline (100 mm Hg) for 20 minutes via a cannula in the left ventricular apex. The heart plus aortic root and descending aorta were excised and stored in 3.7% neutral-buffered formalin (Formal-fixx; Shandon Scientific Ltd, UK). The atherosclerotic lesion areas in oil red O stained cryostat sections of the aortic root were quantified using the Leica image analysis system, consisting of a Leica DMRE microscope coupled to a video camera and Leica Qwin Imaging software (Leica Ltd, Cambridge, UK). Mean lesion area (in μm2) was calculated from 10 oil red O stained sections, starting at the appearance of the tricuspid valves.

Histological Analysis of Lung
Ten-micrometer cryosections of formalin-fixed lungs, of mice that were fed the high-cholesterol diet for 12 weeks, were prepared and used for oil red O staining as described. For the assessment of macrophage content, sections were immunolabeled with a macrophage-specific antigen (MOMA-2, polyclonal rat IgG2b, 1:50 dilution, Research Diagnostics Inc).

Statistical Analysis
Statistical analysis on the macrophage cholesterol efflux studies was performed using the unpaired Student t test (GraphPad InStat and Prism software). Atherosclerotic lesion size of ABCG1+/− and ABCG1−/− mice after 6 and 12 weeks of high-cholesterol diet feeding were tested by 2-factor (time and genotype) analysis of variance (2-way ANOVA) after confirming normal distribution and equal standard deviations, using GraphPad InStat and Prism software. The interaction between time and genotype was also tested. The probability level (alpha) for statistical significance was set at 0.05.

Results

Generation of LDLr−/− Mice Deficient in ABCG1
To investigate the role of macrophage ABCG1 in lipoprotein metabolism and atherogenesis, we used the technique of bone marrow transplantation (BMT) to selectively disrupt ABCG1 in hematopoietic cells. Bone marrow from ABCG1+/− and ABCG1−/− mice was transplanted into LDLr−/− mice, which represent an established model for the development of atherosclerosis. Genomic DNA from bone marrow of transplanted mice was assessed for ABCG1 transcripts to investigate successful reconstitution of recipient mice (Figure 1). Bone marrow from ABCG1+/− → LDLr−/− mice only displayed the 200-kb wild-type-specific band, whereas bone marrow from ABCG1−/− → LDLr−/− mice contained a prominent 380-kb band diagnostic of the disrupted ABCG1 gene and only a faintly detectable band corresponding to the ABCG1 wild-type gene (Figure 1A). Disruption of ABCG1...
The lipoprotein profiles of the 2 groups were essentially similar and no significant differences could be found either (Table).

Before BMT and at the different time points after BMT, no variation in the serum triglyceride levels in both groups of high-cholesterol diet feeding. Although there is more cholesterol efflux from 3H-cholesterol-labeled peritoneal macrophages isolated from ABCG1+/− mice (n=3) or ABCG1−/− mice (n=3), Statistically significant difference (**P<0.001 compared with control).

Effect of Macrophage ABCG1 Disruption in LDLr−/− Mice on Serum Lipid Levels

<table>
<thead>
<tr>
<th>Donor Bone Marrow</th>
<th>Time weeks</th>
<th>Diet</th>
<th>Total Cholesterol (mg/dL)</th>
<th>Triglycerides (mg/dL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>ABCG1+/−</td>
<td>Baseline</td>
<td>Chow</td>
<td>236±8</td>
<td>179±9</td>
</tr>
<tr>
<td></td>
<td>8</td>
<td>Chow</td>
<td>252±12</td>
<td>240±31</td>
</tr>
<tr>
<td></td>
<td>14</td>
<td>WTD</td>
<td>676±93</td>
<td>184±18</td>
</tr>
<tr>
<td></td>
<td>20</td>
<td>WTD</td>
<td>632±67</td>
<td>131±16</td>
</tr>
<tr>
<td>ABCG1−/−</td>
<td>Baseline</td>
<td>Chow</td>
<td>243±8</td>
<td>164±10</td>
</tr>
<tr>
<td></td>
<td>8</td>
<td>Chow</td>
<td>270±16</td>
<td>215±20</td>
</tr>
<tr>
<td></td>
<td>14</td>
<td>WTD</td>
<td>511±66</td>
<td>176±22</td>
</tr>
<tr>
<td></td>
<td>20</td>
<td>WTD</td>
<td>670±56</td>
<td>195±17</td>
</tr>
</tbody>
</table>

Serum lipid levels were measured in LDLr−/− mice before transplantation (baseline) and at 8, 14, and 20 weeks after transplantation with control or bone marrow from ABCG1−/− mice. At 8 weeks after bone marrow transplantation the regular chow diet was switched to a high-cholesterol diet. Data represent mean±SEM of n=8 mice. No statistically significant differences were observed between the control transplanted group and the mice transplanted with ABCG1−/− bone marrow.
of disruption of macrophage ABCG1 on serum lipid levels or lipid distribution among the different lipoproteins was observed.

**Macrophage ABCG1 Disruption and Lipid Homeostasis in the Lungs**

Visual examination of the lungs of ABCG1+/−→LDLr−/− mice showed abnormal lung morphology compared with controls (Figure 3A). Consequently, cryostat sections of the lungs of transplanted animals were prepared and stained for macrophages using the MOMA-2 antigen (Figure 3B) or for lipids using oil red O (Figure 3C, 3D) and counterstained with hematoxylin. Compared with controls an accumulation of macrophages was observed in the subpleural region of the lungs of ABCG1+/−→LDLr−/− mice (Figure 3B). In addition, specifically these macrophages in the subpleural region of the lungs accumulated large amounts of lipids, whereas macrophages in control animals did not accumulate any lipids (Figure 3C, 3D).

**Effect of Macrophage ABCG1 Disruption on Atherosclerotic Lesion Formation**

To investigate the importance of macrophage ABCG1 for atherosclerotic lesion development, we analyzed the aortic root of ABCG1+/−→LDLr−/− and ABCG1+/−→LDLr−/− mice after 6 and 12 weeks of high-cholesterol diet feeding (Figure 4). Representative photomicrographs of the aortic root of control transplanted mice and the mice transplanted with ABCG1+/− bone marrow are shown in Figure 4A. After 6 weeks on the high-cholesterol diet atherosclerotic lesion size in the aortic root was 65±15×10^3 μm^2 in ABCG1+/−→LDLr−/− mice (n=9) versus 49±12×10^3 μm^2 (n=10) in controls and after 12 weeks 167±17×10^3 μm^2 in ABCG1+/−→LDLr−/− mice (n=8) versus 123±18×10^3 μm^2.
macrophages. The abnormal lipid homeostasis in macrophages in the lung of ABCG1−/− mice (this study) and ABCG1−/− mice16 could indicate that macrophage ABCG1 is a specific transporter for lung surfactant lipids and proteins or that it plays a role in the clearance of pulmonary surfactant lipids and proteins.

Because ABCG1 deficiency markedly influenced lipid accumulation in the lungs, we expected also a major effect on atherosclerotic lesion development. Both ABCA1 and ABCG1 are suggested to be involved in RCT and atherosclerosis, because: (1) targeted disruption of ABCA1 or ABCG1 in mice are both associated with accumulation of cholesterol in tissues16,25; (2) ABCA1 and ABCG1 redistribute cholesterol to cell-surface domains, where it can be removed by lipid-free apolipoproteins and lipitated lipoproteins, respectively2–4,11,16; and (3) in macrophages, both ABCA1 and ABCG1 expression is induced on cholesterol loading and LXR activation.5–7,11,13–15 Recently, the athero-protective effects of ABCA1 have been firmly established in animal models. Overexpression of ABCA1 resulted in decreased susceptibility to spontaneous atherosclerosis in apoE−/− mice26 and in C57BL/6 mice with diet-induced atherosclerosis.27 Furthermore, using BMT, we have shown that macrophage-specific inactivation of ABCA1 in LDLr−/− mice after 12 weeks of high-cholesterol diet feeding enhanced atherosclerotic lesion progression, whereas ABCA1 overexpression prevented the progression of atherosclerosis, indicating that macrophage ABCA1 plays an essential protective role in atherosclerosis.28,29

Whereas no effects were observed on serum lipid levels or lipoproteins in the present study, atherosclerotic lesion size was dependent on both time and the macrophage ABCG1 genotype (P = 0.038 by two-way ANOVA) (Figure 4B).

**Discussion**

The availability of ABCG1−/− mice provides an important tool to study the function of the ABCG1 protein. Using these mice, Kennedy et al recently showed that ABCG1 plays a critical role in the prevention of cellular lipid accumulation in the lung.16 On regular chow diet total body ABCG1 deficiency resulted in the accumulation of lipids in macrophages localized to the subpleural region of the lungs in mice aged 15 weeks of age, whereas lipids in other tissues were unaffected. When mice were put on a high-cholesterol/high-fat diet for 9 weeks, cholesterol, triglycerides, and phospholipid concentrations were significantly increased in the livers and lungs of ABCG1−/− mice. In our study, using the method of BMT, we found specifically an accumulation of lipids in macrophages localized to the subpleural region of lungs in ABCG1−/− mice, suggesting that macrophage ABCG1 expression in macrophages in the lung is critically involved in lipid homeostasis in the lung. Several ABC transporters have been implicated in lung pathology.19,20 Alveolar macrophages are involved in the clearance of pulmonary surfactant lipids and proteins21,22 and it has been postulated that the disease of alveolar proteinosis, in which there is an intra-alveolar accumulation of surfactant components, may be caused by defective clearance by macrophages. Interestingly, mice deficient in surfactant protein-D (SP-D)23,24 show an accumulation of alveolar surfactant and an increase in foamy alveolar lesions and counterstained with hematoxylin, are shown (magnification 50×). B, Mean lesion size is shown for LDLr−/− mice transplanted with bone marrow of ABCG1−/− or ABCG1−/− mice after 6 and 12 weeks on high-cholesterol diet (each group n = 8). Atherosclerotic lesion size depended on both time and the macrophage ABCG1 genotype (P = 0.038 by 2-way ANOVA).

Figure 4. Quantitative analysis of atherosclerosis in cross-sections at the aortic root of LDLr−/− mice transplanted with bone marrow of ABCG1−/− or ABCG1−/− mice after 6 and 12 weeks on high-cholesterol diet. A, Representative cross-sections, which were stained with oil red O to visualize lipid-rich lesions and counterstained with hematoxylin, are shown (magnification 50×). B, Mean lesion size is shown for LDLr−/− mice transplanted with bone marrow of ABCG1−/− or ABCG1−/− mice after 6 and 12 weeks on high-cholesterol diet (each group n = 8). Atherosclerotic lesion size depended on both time and the macrophage ABCG1 genotype (P = 0.038 by 2-way ANOVA).
fied gene products may be responsible for the majority of the cholesterol efflux to HDL.

In conclusion, in LDLr−/− mice on a high-cholesterol diet macrophage ABCG1 expression is critically involved in lipid homeostasis in the lung, whereas macrophage ABCG1 moderately, but significantly influenced atherosclerotic lesion development.

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Disclosures

None.

References

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