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 significant 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, deletion 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
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
ABC

Genotyping for ABCG1 was performed according to the protocol from Deltagen Inc. 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 (LDLr) receptor knockout (LDLr−/−) mice were obtained from The Jackson Laboratory, Bar Harbor, Me as mating pairs and bred at the Gorlaeus Laboratories, 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; Abdiets, Woerden, The Netherlands). Drinking water was supplied with antibiotics (83 mg/L cefuroxim 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

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 Andrey Smart 225 Röntgen source (XYLON Int, Copenhagen, Denmark) with a 6-mm aluminum filter. Bone marrow was irradiated 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 0.5×107 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 μCi/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.14 Radioactivity in the medium was determined by 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). Precipitation of lipoproteins in serum was determined by fractionation of 30 μL of serum of each mouse using a Superox6 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-fix; 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 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 P \( \leq 0.001 \) compared with \( \text{HDLr}^{+/+} \) Statistically significant difference ***

In macrophages furthermore resulted in a 22% decrease \((P=0.0007)\) in cholesterol efflux to HDL, whereas efflux to apoAI was not affected (Figure 1B).

### Effect of Macrophage ABCG1 Disruption on Plasma Lipid Levels

During the weeks after BMT, the total serum cholesterol and triglyceride levels were repeatedly determined (Table). No significant differences in lipid concentrations between ABCG1 \(^{+/+}\) → LDLr \(^{-/-}\) mice and ABCG1 \(^{-/-}\) → LDLr \(^{-/-}\) mice could be observed at 8 weeks after BMT and levels were not significantly different from those measured at baseline, before BMT. To induce atherosclerotic lesion formation, the transplanted LDLr \(^{-/-}\) mice were fed a high-cholesterol diet containing 0.25% (wt/wt) cholesterol and 15% (wt/wt) fat starting at 8 weeks after BMT. As a result, the total serum cholesterol levels in both the control and experimental groups increased \( \approx 3\)-fold but also under these conditions no differences were observed between the ABCG1 \(^{+/+}\) → LDLr \(^{-/-}\) mice and ABCG1 \(^{-/-}\) → LDLr \(^{-/-}\) mice after 6 and 12 weeks of high-cholesterol diet feeding. Although there is more variation in the serum triglyceride levels in both groups before BMT and at the different time points after BMT, no significant differences could be found either (Table).

The distribution of cholesterol and triglycerides among serum lipoproteins was analyzed by liquid chromatography. The lipoprotein profiles of the 2 groups were essentially identical on both the standard chow diet (Figure 2A, week 8 after BMT) and the high-cholesterol diet (Figure 2B and Figure 2C, week 14 and week 20 after BMT, respectively). After the switch to high-cholesterol diet, cholesterol in the very LDL and intermediate density lipoprotein/LDL fraction increased \( \approx 6\)-fold, whereas there was no significant change of cholesterol in the HDL fraction. Thus, no significant effect

<table>
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<th>Donor Bone Marrow</th>
<th>Time weeks</th>
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<th>Triglycerides (mg/dL)</th>
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<td>ABCG1 (^{+/+})</td>
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<td>Chow</td>
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<td>179±9</td>
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<tr>
<td></td>
<td>8</td>
<td>Chow</td>
<td>252±12</td>
<td>240±31</td>
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<tr>
<td></td>
<td>14</td>
<td>WTD</td>
<td>676±93</td>
<td>184±18</td>
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<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>
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<td>270±16</td>
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<tr>
<td></td>
<td>20</td>
<td>WTD</td>
<td>670±56</td>
<td>195±17</td>
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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 \( \approx 8\) mice. No statistically significant differences were observed between the control transplanted group and the mice transplanted with ABCG1 \(^{-/-}\) bone marrow.

**Figure 1.** Verification of success of bone marrow transplantation. A, Verification of successful reconstitution with donor hematopoietic cells by polymerase chain reaction amplification of ABCG1 wild-type and targeted alleles at 20 weeks after transplantation using genomic DNA isolated from bone marrow. A multiplex reaction was performed to detect both wild-type (200 bp) and targeted (381 bp) alleles, whereas a second reaction was performed to detect only the wild-type or endogenous allele. Molecular weight marker: 1 \( \approx 500\) bp, 2 \( \approx 400\) bp, 3 \( \approx 300\) bp, and 4 \( \approx 200\) bp. B, ApoAI and HDL induced cellular cholesterol efflux from \( ^{3}H\)-cholesterol-labeled peritoneal macrophages isolated from ABCG1 \(^{+/+}\) mice (n=3) or ABCG1 \(^{-/-}\) mice (n=3). Statistically significant difference ***P<0.001 compared with control.

**Figure 2.** Distribution of serum cholesterol and triglycerides over the different lipoprotein fractions in LDLr \(^{-/-}\) mice transplanted with ABCG1 \(^{+/+}\) or ABCG1 \(^{-/-}\) bone marrow. Blood samples were drawn by tail bleeding after an overnight fasting period. A 30-μL aliquot of serum of each individual mouse was loaded onto a Pharmacia Smart column and fractions were collected. Fractions 3 to 6 represent very LDL and chylomicrons, fractions 8 to 12 represent LDL, and fractions 16 to 20 represent HDL. The distribution of cholesterol before chow diet (A) and after a high-cholesterol diet (B) over the different lipoproteins in ABCG1 \(^{+/+}\) → LDLr \(^{-/-}\) mice (black squares) and ABCG1 \(^{-/-}\) → LDLr \(^{-/-}\) mice (white squares) is shown. Values are the means of \( \approx 8\) mice for each group. No significant differences in the distribution of serum cholesterol and triglycerides was observed between the 2 groups.
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<sup>+/−</sup>→LDLR<sup>/−/−</sup> 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<sup>/−/−</sup>→LDLR<sup>/−/−</sup> 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<sup>+/+</sup>→LDLR<sup>/−/−</sup> and ABCG1<sup>/−/−</sup>→LDLR<sup>/−/−</sup> 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<sup>/−/−</sup> 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<sup>3</sup> μm<sup>2</sup> in ABCG1<sup>/−/−</sup>→LDLR<sup>/−/−</sup> mice (n=9) versus 49±12×10<sup>3</sup> μm<sup>2</sup> (n=10) in controls and after 12 weeks 167±17×10<sup>3</sup> μm<sup>2</sup> in ABCG1<sup>/−/−</sup>→LDLR<sup>/−/−</sup> mice (n=8) versus 123±18×10<sup>3</sup> μm<sup>2</sup>.
found specifically an accumulation of lipids in macrophages (F = 0.038 by 2-way ANOVA). Atherosclerotic lesion size depended on both time and the ABCG1 genotype (P = 0.038 by 2-way ANOVA).

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 ≥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−/−→LDLr−/− 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 macrophages. The abnormal lipid homeostasis in macrophages in the lung of ABCG1−/−→LDLr−/− 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, respectively2–4,11,13; 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−/− mice27 and in C57BL/6 mice with diet-induced atherosclerosis.28 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.29,30

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). The absence of macrophage ABCG1 led to an increase in lesion formation of 33% to 36%. Macrophage ABCA1 deficiency, however, did lead to a 2-fold increase in lesion development in LDLr−/− mice after both 6 (unpublished results) and 12 weeks on a high-cholesterol diet,9 establishing that at both time points the potential effect of ABCG1 deficiency on atherosclerotic lesion formation is certainly less prominent as compared with ABCA1.

In vitro we observed a significant effect of ABCG1 deficiency on cholesterol efflux to HDL even for macrophages not stimulated for LXR, whereas Kennedy et al did only observe a significant effect under conditions of LXR activation.16 In agreement with Kennedy et al we did not observe any effect of ABCG1 deficiency with apoAI as cholesterol acceptor.16 Wang et al recently showed that LXR activation significantly increased cholesterol mass efflux to HDL from acetylated LDL-loaded macrophages of ABCG1 wild-type mice, whereas the increased cholesterol mass efflux was markedly reduced in ABCG1-deficient macrophages.28 Basal cholesterol mass efflux was however only slightly decreased by ABCG1 deficiency.28 In contrast, ABCA1 deficiency or overexpression does specifically modulate cholesterol efflux to apoAI, whereas SR-BI and ABCG1 are utilizing HDL as acceptor.5,10,16,29 The absence of SR-BI or ABCG1 only affect cholesterol release to HDL for 20%29 and 22% (this study), respectively, indicating that still unidenti-
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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