Decreased Atherosclerosis in Low-Density Lipoprotein Receptor Knockout Mice Transplanted With Abcg1−/− Bone Marrow

Mollie Ranalletta, Nan Wang, Seongah Han, Laurent Yvan-Charvet, Carrie Welch, Alan R. Tall

Objective—Recent studies indicate that the ATP-binding cassette transporter ABCG1 can promote cholesterol efflux from macrophages to high-density lipoprotein. This study was designed to assess the in vivo role of macrophage ABCG1 in atherosclerosis.

Methods and Results—Bone marrow from Abcg1−/− mice was transplanted into irradiated Ldlr−/− recipients, and atherosclerosis was evaluated by aortic root assay after 7 or 11 weeks of feeding on a Western diet. After 7 weeks, there was no difference in lesion area in mice receiving either wild-type or Abcg1−/− bone marrow, whereas after 11 weeks, lesion area was moderately but significantly reduced in Abcg1−/− recipients. ABCG1-deficient peritoneal macrophages showed induction of several liver X receptor target genes, such as Abca1 and Srebp1c, and a dramatic increase in apolipoprotein E (apoE) protein both in cell media and lysates, without parallel change in apoE mRNA. Abca1 knockout prevented the increase in apoE secretion but had minimal effects on apoE accumulation in cell lysates of Abcg1−/− macrophages. Plasma apoE levels were markedly increased in recipients of Abcg1−/− bone marrow.

Conclusions—These studies reveal an inverse relationship between Abcg1 expression and apoE accumulation and secretion in macrophages. The reduced atherosclerosis in recipients of Abcg1-deficient bone marrow may be explained by induction of Abca1 and an associated increase in macrophage apoE secretion. (Arterioscler Thromb Vasc Biol. 2006; 26:2308-2315.)

Key Words: atherosclerosis ■ ABC transporters ■ apoE ■ macrophages

Plasma high-density lipoprotein (HDL) levels have an inverse relationship to atherosclerotic cardiovascular disease. HDL has a variety of antiinflammatory, antioxidant, antithrombogenic, and vasodilatory properties that may be relevant to this relationship.1,2 The ability of HDL to promote cholesterol efflux from cholesterol-loaded macrophage foam cells is thought to be of central importance in its antiatherogenic properties. Consistent with this idea, patients with Tangier disease and mutations in the ATP-binding cassette transporter ABCA1, have defects in cellular cholesterol efflux, very low HDL levels, prominent foam cell accumulation in tissues, and premature atherosclerosis.3–5 Cholesterol-loaded macrophages show increased expression of ABCA1, as a result of activation of the transcription factors liver X receptor/retinoid X receptor (LXR/RXR) by endogenous oxysterols accumulating within cells.6–9

ABCA1 mediates cholesterol efflux to lipid-poor apoA-1 but only modestly increases cholesterol efflux to HDL-3 and does not increase cholesterol efflux to HDL-2 particles.10 Recently, we and others have shown, by both overexpression and genetic knockdown approaches, that ABCG1 promotes the efflux of cholesterol from macrophages to HDL particles but not to lipid-poor apoA-1.10–12 This activity appears to be particularly prominent following macrophage LXR activation, which induces both Abcg1 gene expression, as well as translocation of ABCG1 from intracellular sites to the plasma membrane.13 Abcg1−/− mice did not show obvious changes in their plasma lipoprotein profiles but when placed on a high cholesterol diet, demonstrated subpleural accumulation of foam cells in their lungs and Oil Red O–positive lipid droplets in their hepatocytes, suggesting that ABCG1 defends against macrophage and hepatocyte neutral lipid accumulation in vivo.11 Conversely, mice overexpressing human ABCG1 were protected from lipid accumulation in liver and lungs when fed a high-fat diet.11 Peritoneal macrophages isolated from diabetic db/db mice had reduced ABCG1 expression, impaired cholesterol efflux to HDL, and accumulation of esterified cholesterol.14 These observations suggested that macrophage ABCG1 might decrease foam cell formation in arteries and have antiatherogenic properties in vivo. Although general deficiency of ABCG1 does not induce atherosclerosis, it is possible that ABCG1 deficiency in...
macrophages worsens atherosclerosis in a susceptible background. Therefore, to evaluate a possible proatherogenic effect of macrophage ABCG1 deficiency, we carried out bone marrow (BM) transplantation from Abcg1−/− mice into Ldlr−/− mice fed a Western diet.

**Methods**

**Animals and Diets**

Abcg1−/− mice were purchased from DeltaGen and backcrossed 5 generations into the C57Bl/6j background. Ldlr−/− mice were purchased from The Jackson Laboratory (Bar Harbor, Me). All mice were housed at Columbia University Medical Center according to animal welfare guidelines. Animals had ad libitum access to both food and water. Mice were fed chow (Purina Mills diet 5053) or Western diet (42% from fat adjusted calorie diet, TD 88137, Harlan Teklad).

**BM Transplant**

BM transplantation was performed as previously described. Mice were allowed to recover for 5 weeks after irradiation and BM transplantation before diet studies were initiated. During this recovery phase, peripheral blood was collected and DNA isolated to determine the efficiency of BM reconstitution by quantification of wild-type ABCG1 compared with actin.

**Tissue Collection Aortic Lesion Analysis**

Mice were euthanized in accordance with the American Veterinary Association Panel on Euthanasia. Lesion analysis was performed as previously described. The average from 5 sections for each animal was used to determine lesion size.

**Plasma Cholesterol, Triglyceride, and Lipoprotein Measurements**

Blood samples were collected by retro-orbital venous plexus puncture after a 5- to 6-hour fast. Plasma was separated by centrifugation and stored at −70°C. Total plasma cholesterol and triglyceride was determined enzymatically using kits from Wako. Pooled plasma (250 µL) from 6-hour fasted mice (n=15 mice per each group) was used for fast protein liquid (FPLC) analysis using 2 Superose 6 columns in series. The buffer contains 0.1 mol/L Tris-HCL and 0.4% NaN3, and a flow rate of 0.7 mL/min was used.

**Western Blot Analysis**

The expression of ABCG1, ABCA1, apoE, and β-actin were measured in pooled peritoneal macrophage cells by Western blot analysis. Primary antibodies for ABCG1 and ABCA1 were purchased from Santa Cruz Biotechnology Inc (Santa Cruz, Calif) or Novus Biologicals (Littleton, Colo), respectively. HDL cholesterol (HDL-C) was isolated by chemical precipitation and cholesterol content determined enzymatically using kits from Wako. Pooled plasma (250 µL) from 6-hour fasted mice were treated for 16 hours, and the media or cell lysates were collected respectively. Equal volume of media normalized against cellular proteins and equal amount of cellular proteins (30 µg) from each sample were used for Western blot analysis. Small interfering RNA (siRNA) experiments were performed as described previously. Briefly, mouse peritoneal macrophages from the wild-type or Abcg1−/− mice were treated with the scrambled or ABCA1 siRNA for 32 hours. Then the cells were washed and the media were replaced with fresh DMEM media containing 0.2% bovine serum albumin in the presence or absence of 2 µmol/L TO901317 plus 50 µg/mL acLDL protein. Cells were treated for 16 hours, and the media or cell lysates were collected for Western blot analysis. The cholesterol efflux assay was performed as previously described using 50 mg/mL acLDL and 3 mmol/L TO901317. Cholesterol efflux was measured 4 hours after incubation with or without apoA-1.

**RNA Analysis**

Mouse peritoneal macrophages were harvested and cultured for 2 hours in DMEM media containing 10% FBS. Total RNA was isolated from mouse peritoneal macrophages using the RNeasy kit (Qiagen, Valencia, Calif) according to the instructions of the manufacturer. Real-time quantitative polymerase chain reaction assays were performed as previously described. All samples were analyzed for β-actin RNA expression in the same run as ABCA1, ABCG1, sterol regulatory element binding protein (SREBP)-1c, apoE, or phospholipid transfer protein (PLTP). The sequences of the probes and primers can be provided by the authors on request.

**Statistical Analysis**

Statistical analysis was determined using unpaired, 2-tailed, Student’s t test. Results are expressed as an average ± SE.

**Results**

**Atherosclerosis Studies in Ldlr-Deficient Mice Transplanted With Abcg1+/+ or Abcg1−/− BM**

To evaluate the impact on atherogenesis of ABCG1 in BM-derived cells, BM was transplanted from male Abcg1−/− mice into female Ldlr−/− recipients. Four weeks after transplantation, Abcg1 gene content in whole blood was reduced more than 89% compared with wild-type BM recipients (Figure 1 in the online data supplement, available at http://atvb.ahajournals.org); both groups achieved similar levels of engraftment of donor BM as indicated by Y chromosome content in whole blood (supplemental Figure I). Animals were placed on the Western diet and euthanized after 7 or 11 weeks for atherosclerosis quantification. Western blot analysis of peritoneal macrophages obtained from mice during the atherosclerosis study showed no detectable ABCG1 in Abcg1−/− BM recipients, confirming the efficiency of the transplantation procedure (see below). There was no difference in the extent or appearance of atherosclerotic lesions in the proximal aorta after 7 weeks on the diet (Figure 1A and 1C). Unexpectedly, atherosclerotic lesion area was moderately and significantly reduced in Abcg1−/− BM recipients after 11 weeks on the diet (Figure 1B and 1C). The lesion content of macrophages and smooth muscle cells, as shown by Mac-3 or actin staining, was the same in both groups (mac-3: control, 84.6±2.5%; Abcg1−/−), 77.8±3.8%; actin: control, 15.4±2.5%; Abcg1−/−, 22.2±3.8%). We undertook further studies to define the mechanism of decreased atherosclerosis in Abcg1−/− BM recipients.

**Plasma Lipoprotein Analysis**

Plasma lipoprotein analysis by precipitation methods revealed a small decrease in the total and non-HDL (ie, very-low-density lipoprotein [VLDL]/LDL) cholesterol levels in Abcg1−/− BM recipients on the chow diet (Table). After 7 weeks on the Western diet, plasma cholesterol levels were
increased in both groups and there was no significant difference in VLDL/LDL cholesterol levels in the Abcg1/−/− recipients, compared with the controls. There were no changes in HDL-C levels at either time point. Analysis of the plasma lipoprotein profile by FPLC showed only a slight decrease in VLDL and LDL cholesterol levels in the Abcg1/−/− BM recipients, with no difference in HDL levels (supplemental Figure II). After 11 weeks on the Western diet, plasma cholesterol levels had risen further and there was no difference in lipoprotein levels between the groups. These findings indicated that changes in plasma lipoprotein levels could not account for the decrease in atherosclerosis at the 11 week time-point.

Changes in Gene Expression in ABCG1-Deficient Macrophages

We next considered the possibility that deficiency of ABCG1 in macrophages might lead to compensatory induction of other genes with antiatherogenic consequences. Peritoneal macrophages were harvested from a subgroup of transplanted mice after 7 or 11 weeks on the Western diet. There was a 2-fold induction of ABCA1 mRNA in macrophages from Abcg1/−/− BM recipients compared with controls at 7 weeks and a 1.2-fold induction at 11 weeks (P=0.005) (Figure 2A and 2B). Western blots confirmed the absence of ABCG1 and an increase in ABCA1 protein levels in Abcg1/−/− macrophages (Figure 2C). Another LXR target gene, Srebp1c, was also induced at 7 weeks (1.8-fold) and at 11 weeks (1.2-fold) (Figure 2A and 2B). Activity of the Abcg1 promoter was monitored by measuring a 5′ transcript, upstream of the targeted region. This also showed an induction of expression, 3.2-fold at 7 weeks and 1.2-fold at 11 weeks (Figure 2A and 2B). Thus, there was induction of several different LXR targets in Abcg1/−/− macrophages. In macrophages PLTP and apoE are also LXR targets, but changes in expression tend to be less than that of Abca1 and Abcg1.6,7,17–19 In contrast to the ABC transporters and Srebp1c, apoE, PLTP, 3-hydroxy-3-methylglutaryl–coenzyme A reductase, and acyl-coenzyme A acyltransferase (ACAT) mRNAs were not significantly increased at 7 or 11 weeks (Figure 2A and 2B).

Increased ApoE Protein in Cells and Media of Abcg1/−/− Macrophages

Macrophage apoE has multiple potent antiatherogenic functions.20–26 Even though apoE mRNA was not increased, we wished to evaluate the possibility that apoE secretion might be changed in ABCG1-deficient macrophages. Indeed we discovered that apoE levels in cell media were increased from 1.6- to 6.3-fold in Abcg1/−/− macrophages compared with wild-type cells (Figure 3). The increase of apoE in media was more prominent in cells treated with the LXR activator TO901317, and in cells treated with acLDL+TO901317, apoE appeared at higher molecular weight, suggesting increased glycosylation.27–29 ApoE was also increased in cell lysates of Abcg1/−/− macrophages, and this effect was prominent under basal conditions (3.9-fold) as well as after treatment with T0+acLDL (7.3-fold) (Figure 3). As expected, ABCA1 was...
protein was also increased in ABCG1-deficient cells, and this increase was more prominent after acLDL loading or LXR activation (3 to 4 fold). Similar results were obtained for 3 different preparations of macrophages. Knockdown of ABCG1 expression in wild-type macrophages by siRNA resulted in a similar increase in ABCA1 protein and in apoE secretion (supplemental Figure III), indicating that these changes were cell autonomous. Because ABCG1 was only partly reduced by siRNA (~70%), these findings indicate that increased apoE secretion is also observed in cells that only have a partial deficiency of ABCG1.

**Knockdown of ABCA1 Reduces ApoE in Macrophage Media but Not in Cells**

We next carried out experiments to determine the mechanism of increased apoE accumulation in media of ABCG1-deficient cells. Some but not all earlier studies have shown that apoE secretion is decreased in ABCA1-deficient cells.30–32 To evaluate the possibility that increased apoE accumulation in media might be secondary to the increase in ABCA1 in Abcg1−/− cells, we carried out siRNA knockdown of ABCA1 in Abcg1−/− cells. The knockdown of ABCA1 was highly effective (Figure 4A). This led to a marked decrease in apoE accumulation in media of wild-type cells (0.6-fold) and an even more prominent decrease in Abcg1−/− cells (0.3- to 0.4-fold). The knockdown of ABCA1 resulted in an almost complete reversal of the increase of apoE in media of Abcg1−/− cells compared with wild-type cells (Figure 4A).

Interestingly, in contrast to the effects on ABCA1 knockdown on apoE in media, the increase in apoE in cell lysates in Abcg1−/− cells was minimally affected by knockdown of ABCA1 by siRNA (Figure 4A). Knockdown of ABCA1 by siRNA also resulted in a marked decrease in apoE in media but not in cell lysates under the other treatment conditions (acLDL or T0 alone, data not shown). In summary, these data suggest that the induction of ABCA1 in ABCG1-deficient cells is primarily responsible for increased apoE secretion but indicate that an additional novel mechanism is responsible for the increase of apoE in ABCG1-deficient cells.

**Increased ApoE in Plasma of Abcg1−/− BM Recipients**

To determine whether increased secretion of apoE by BM-derived cells might be occurring in vivo, we measured apoE by Western blotting in plasma samples. This showed significant increases in apoE levels in plasma from Abcg1−/− BM recipient mice compared with wild type, both under basal conditions (2.7-fold) and after feeding the
Western diet (≈2-fold) (Figure 4B). The prominently increased apoE on the chow diet likely explains the decrease in non–HDL-C on this diet (Table). The Western diet did increase plasma apoE levels in both groups (not shown, Figure 4B shows different dilutions of samples for chow and Western diets), and the fold increase attributable to ABCG1 deficiency became less pronounced. These findings strongly suggest increased secretion of apoE by BM-derived cells caused by ABCG1 deficiency in vivo as well as in cell culture.

**Decreased Cholesterol Efflux to ApoA-1 in Abcg1−/− Macrophages**

To determine whether the increase in apoE secretion in Abcg1−/− macrophages was associated with an increase in cholesterol efflux, peritoneal macrophages were loaded with 3H-cholesterol-acLDL and then 3H-cholesterol efflux to media alone (DMEM/0.1% BSA) or media containing 10 μg/mL apoA-1 was determined. Cholesterol efflux to media alone was very low and was slightly increased in Abcg1−/− cells (Figure 5). Even though ABCA1 was increased in Abcg1−/− cells, cholesterol efflux to apoA-1 was significantly reduced by ≈25% in Abcg1−/− cells (Figure 5). This could be because ABCG1 normally acts on lipidated apoA-1 particles following the action of ABCA1, and this activity is lost in Abcg1−/− cells.

**Discussion**

Unexpectedly, a deficiency of ABCG1 in BM-derived cells did not lead to an increase in atherosclerosis at 7 weeks in Ldlr−/− mice fed Western diet and was even associated with a significant decrease in lesion area at 11 weeks. These findings appear to be explained by a compensatory increase in ABCA1 mRNA and protein levels and a marked increase in apoE secretion in ABCG1-deficient macrophages.

The induction of ABCA1 expression and the increase in apoE secretion in macrophages of mice transplanted with Abcg1−/− BM is a plausible mechanism to explain the atherosclerosis results. Similar to our results, in a BM transplantation experiment carried out in Ldlr−/− mice fed the Western diet, moderate overexpression of ABCA1 in BM-derived cells caused by an Abca1 BAC transgene led to no change in atherosclerosis at 9 weeks and a moderate decrease at 12 and 15 weeks. A prominent increase in plasma apoE levels in Abcg1−/− BM recipients suggested that an increased secretion of apoE by myeloid cells occurred in vivo. There is abundant evidence that macrophage apoE expression acts via a variety of different mechanisms to reduce atherosclerosis. Although apoE has been reported to increase macrophage cholesterol efflux, potentially by both ABCA1-dependent and -independent mechanisms, cholesterol efflux was impaired in Abcg1−/− macrophages, despite the increase in ABCA1 and apoE secretion. ApoE enhances the clearance
of VLDL and LDL from plasma,23,25,34 but the changes in plasma lipoprotein levels are too small to explain our atherosclerosis results. ApoE can also have antiinflammatory effects in the vessel wall24 and has been shown to suppress Type I mediated inflammatory–mediated inflammatory responses.35 A modest increase in macrophage apoE in BM cells reduced atherosclerosis susceptibility without affecting plasma lipid levels.36 Moreover, low level expression of apoE in the adrenal resulted in reduced atherosclerosis even in the absence of changes in plasma lipoproteins.37 Thus, the antiinflammatory properties of apoE may be responsible for the reduction in atherosclerosis observed in the Abcg1<sup>−/−</sup> BM recipients of our study.

These studies have uncovered a striking inverse relationship between Abcg1 expression and apoE accumulation and secretion by macrophages. ApoE secretion was increased several fold, whereas apoE mRNA was minimally changed in ABCG1-deficient cells. The increase in apoE in media of ABCG1-deficient cells was primarily driven by increased Abca1 and likely represents increased secretion and/or decreased degradation of apoE as a result of lipidation of endogenous macrophage apoE by ABCA1. A major fraction of macrophage apoE is degraded intracellularly.38 Our findings are consistent with some earlier reports that ABCA1 deficiency is associated with decreased apoE secretion by macrophages30 and further demonstrates a profound effect of Abca1 expression on apoE secretion over a wide range of Abca1 expression levels. There is some evidence that LXR activation increases apoE recycling from intracellular sites, leading to reduced degradation and increased secretion,39 and present findings confirm the speculation that this is likely secondary to induction of ABCA1.

An even more striking finding was a marked accumulation of apoE in Abcg1<sup>−/−</sup> cell lysates, which was observed under all treatment conditions and was minimally affected by knockdown of ABCA1 expression. These findings suggest that ABCG1 activity normally enhances entry of apoE into cellular degradation pathways. ABCG1 appears to reside in the Golgi, recycling endosomes, and plasma membrane.13 Perhaps ABCG1 activity leads to entry of endosomal apoE into a default trafficking pathway evventing in lysosomes, rather then recycling through the endosomal recycling compartment. In ABCG1-deficient cells, increased recycling of apoE from endosomes to Golgi could lead to increased levels of sialation accounting for the high-molecular-weight forms of apoE observed in some experiments (Figure 3).

The induction of Abca1, SREBP1c, and Abcg1 in ABCG1-deficient cells could result from alterations in cellular cholesterol homeostasis that lead to activation of endogenous LXRs. ABCG1 appears to promote cholesterol movement from the endoplasmic reticulum and possibly other intracellular sites to the plasma membrane, where it becomes available for efflux to a variety of acceptors.13 Accumulation of cholesterol in the endoplasmic reticulum or other intracellular organelles may lead to increased formation of endogenous LXR activators. Thus there could be accumulation of an endogenous high-affinity LXR ligand that is formed in increased amounts in ABCG1-deficient cells. Curiously, although the induction of Abca1 was seen under all cell conditions, it appeared most prominent in the context of LXR activation by T0 or acLDL (Figure 3). Thus an alternative explanation is that ABCG1 activity decreases the effectiveness of LXR ligands, by causing a change in their intracellular distribution or secretion.

Even though ABCA1 and apoE secretion were both increased in Abcg1<sup>−/−</sup> macrophages, cholesterol efflux to HDL and apoA-1 was reduced in these cells13 (Figure 5), and cholesterol efflux to acceptor-free media was insignificant. Even though ABCG1 does not directly promote cholesterol efflux to apoA-1,10–12 sequential exposure of apoA-1 to ABCA1 then ABCG1 results in a higher level of cholesterol efflux than exposure to ABCA1 alone, presumably because ABCA1 adds lipids to apoA-1, forming an HDL particle that can then interact with ABCG1.41 Such cooperation of ABCA1 and ABCG1 in wild-type cells
likely explains why cholesterol efflux to apoA-I occurs at a lower level in Abcg1−/− macrophages (Figure 5). A surprising finding was that despite a moderate global defect in cholesterol efflux properties of Abcg1−/− macrophages, and accumulation of foam cells in sites where tissue macrophages are normally found in cholesterol-fed Abcg1−/− mice, the propensity to foam cell formation is not sufficient to cause an increase in atherosclerosis. This indicates that additional properties of macrophages, for example their migratory or inflammatory responses, must be altered to make them accumulate in the vessel wall.

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Disclosures
None.

References


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**Supplemental Figure 1.** *Abcg1* and Y chromosome content in whole blood from *Ldlr*−/− mice receiving *Abcg1*−/− (n=14) or WT (n=14) bone marrow. DNA content was determined by real time quantitative PCR. Both *Abcg1* and Y chromosome content were normalized to actin. * indicates P<0.0001 when comparing mice with WT or *Abcg1*−/− BM.

**Supplemental Figure 2.** FPLC analysis of plasma cholesterol from WT or *Abcg1*−/− BM recipients fed Western diet for 7wks.

**Supplemental Figure 3.** Mouse peritoneal macrophages were treated with either scrambled siRNA (S) or siRNA targeting ABCG1 (G1) for 32 hours, followed by treatment for 16 hrs in the presence or absence of 2 µM TO901317. Media or cell lysates were collected for Western analysis.
Supplemental Figure 1

DNA content

- Abcg1
- Y Chromosome

Legend:
- Control
- Abcg1-/-
Supplemental Figure 2

A.

Cholesterol (mg/dl)

WT
Abcg1-/-
Supplemental Figure 3

<table>
<thead>
<tr>
<th>TO901317</th>
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ABCG1 (Cell)

ABCA1

β-actin

ApoE (Medium)