Total Body ABCG1 Expression Protects Against Early Atherosclerotic Lesion Development in Mice

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Objective—ABCG1 has recently been identified as a facilitator of cholesterol and phospholipid efflux from macrophages to HDL. In bone marrow transplantation studies, we and others have now shown that the absence of macrophage ABCG1 may differentially influence atherosclerotic lesions dependent on the experimental setting and/or the stage of atherosclerotic lesion development. To further define the role of ABCG1 in atherogenesis, we investigated in the current study the effect of total body deficiency of ABCG1 on atherosclerotic lesion development.

Methods and Results—ABCG1−/− mice and wild-type littermates were fed an atherogenic diet for 12 weeks to induce atherosclerotic lesion formation. Both before and after the start of the atherogenic diet, serum lipid levels and lipoprotein profiles did not differ significantly between the two groups. In addition no significant difference in serum apoE levels was found after diet feeding. In wild-type mice the atherogenic diet induced the formation of macrophage-rich early lesions (size: 24±7×10³ μm² [n=6]). Feeding ABCG1−/− mice the atherogenic diet led to a significant 1.9-fold stimulation of atherosclerotic lesion size (46±6×10³ μm² [n=7]; Student t test P=0.034 and Mann–Whitney test P=0.050) compared with controls, suggesting a clear antiatherogenic role for ABCG1. At the same time, excessive lipid accumulation was observed in macrophage-rich areas of the lungs and spleens of ABCG1−/− mice as compared with wild-type mice.

Conclusions—Total body ABCG1 expression protects against early atherosclerotic lesion development. (Arterioscler Thromb Vasc Biol. 2007;27:594-599.)

Key Words: ABCG1 deficiency ■ atherosclerosis ■ atherogenic diet ■ lung ■ spleen ■ mice

Lipid loading of macrophages attributable to excessive uptake of circulating (modified) LDL is a decisive process in the formation of atherosclerotic lesions. Several ATP-binding cassette (ABC) transporters have been shown to play important roles in macrophage lipid metabolism and atherosclerosis (reviewed by Schmitz et al and Van Eck et al). The half-transporter ABCG1 is highly induced in lipid-laden macrophages where it is able to facilitate cellular cholesterol and phospholipid efflux to HDL. As cholesterol efflux from macrophages results in decreased cellular lipid loading, macrophage ABCG1 expression has been suggested to protect against atherosclerosis. Evidence for this concept was provided by earlier studies on ABCA1, a cholesterol/phospholipid efflux mediator to lipid-poor apoproteins like apoAI or ApoE, whereby macrophage expression protected against early atherosclerotic lesion formation, while overexpression inhibited lesion progression. The evaluation of the critical role of macrophage ABCA1 in lesion formation and progression was based on bone marrow transplantation studies, using mostly LDL receptor–deficient mice as recipients with ABCA1-deficient mice or ABCA1 overexpressor mice as donors leading to the anticipated induction and inhibition of lesion formation, respectively. For reason that, similarly to ABCA1, ABCG1 is able to efflux cholesterol from macrophages, it was anticipated that ABCG1 would add to the protective function of ABCA1 in lesion formation. This hypothesis was strengthened by data from Kennedy et al who showed that administration of a high fat/high cholesterol diet to ABCG1-deficient mice resulted in the massive accumulation of lipids in tissue macrophages, whereas overexpression of human ABCG1 protected the murine tissues from dietary fat-induced lipid accumulation.

Recently, 3 groups reported transplantation studies of ABCG1-deficient (ABCG1−/−) bone marrow into LDL receptor–deficient mice with apparently contrasting effects on atherosclerotic lesion formation. In agreement, both Out et al and Baldan et al observed severe lipid deposition in the macrophages of the lungs of LDL receptor–deficient mice receiving ABCG1−/− bone marrow. Out et al showed in LDL receptor–deficient mice a moderate protection of macrophage ABCG1 on atherosclerotic lesion formation (33% to 36%) both at 6 and 12 weeks on a Western-type diet containing...
15% fat and 0.25% cholesterol. In contrast, Báldán et al observed an increase in lesion formation of 35% to 40% with mice fed a diet with 21% fat and 1.25% cholesterol for 12 weeks, whereas Ranalletta et al found in mice on a 42% fat diet at 7 weeks no effect on lesion formation and at 11 weeks a 32% increase in lesion area in the presence of macrophage ABCG1. These different results are discussed in an editorial by Curtiss.

The 33% to 36% increase in lesion formation in the absence of ABCG1 as observed by Out et al is in agreement with the suggested and demonstrated role of ABCG1 in cholesterol efflux. The paradoxal decrease in lesion formation as observed by Báldán et al is explained by increased apoptosis of ABCG1-deficient macrophages, whereas Ranalletta et al explained the moderately reduced lesion formation at 11 weeks on the diet in ABCG1−/− recipients by an induction of ABCA1 and increased apoE levels. Both the induced apoptosis and increased efflux mechanisms will be dependent on serum cholesterol levels which were for the Báldán et al study around 1040 mg/dL, for the Ranalletta et al study around 1170 mg/dL at 11 weeks of diet, while with our mild 0.25% cholesterol/15% fat diet at 12 weeks we reached a value of 670 mg/dL.

To provide a possible explanation for the apparently contrasting effects of ABCG1 deficiency on atherosclerotic lesion formation, we performed an additional study subjecting the ABCG1−/− mice backcrossed 7 times on a C57Bl/6 background to a 15% fat, 1% cholesterol, and 0.5% cholate (ATHERO) diet for 12 weeks. Data represent means ± SEM of 6–7 mice.

### Materials and Methods

#### Animals

ABC1−/− mice, obtained from Deltagen Inc, San Carlos, California, and back-crossed on a C57Bl/6 background for 7 generations, were cross-bred to generate ABCG1+/+ and ABCG1−/− mice. Genotyping for ABCG1 was performed according to the protocol from Deltagen. Mice were maintained on sterilized regular chow containing 4.3% (w/w) fat and no cholesterol (RM3, Special Diet Services).

At 12 weeks of age, the diet was switched to a semisynthetic atherogenic diet containing 15% (w/w) fat, 1% (w/w) cholesterol, and 0.5% cholate (Diet N, Abdiets). After 12 weeks of feeding the diet, the mice were fasted overnight. Subsequently, a whole-body perfusion was performed using phosphate-buffered saline containing 1 mmol/L EDTA (4°C, 100 mm Hg) for 20 minutes. After perfusion, organs were excised and stored in 3.7% formalin until further use. 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.

#### Serum Lipid Analyses

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), and 15 µg/mL cholesteryl esterase (Roche Diagnostics) in reaction buffer (1.0 KPi buffer, pH = 7.7 containing 0.01 mol/L phenol, 1 mmol/L 4-aminooantipyrine, 1% polyoxyethylene-9-lauryl ether, and 7.5% methanol). Absorbance was read at 490 nm. Precipath (standardized serum; Roche) was used as internal standard. The distribution of cholesterol over the different lipoproteins in serum was determined with an ImmunoCAP (Pharmacia) system using an anti-cholesterol antibody and an internal control.

### Results

#### Absence of ABCG1 Does Not Alter Serum Lipid and ApoE Levels

On a chow diet containing 4.3% fat and no cholesterol, no significant differences in body weight and lipid concentrations between ABCG1+/+ mice and ABCG1−/− mice could be observed (Table). Subsequently, to induce atherosclerotic lesions, the mice were fed a diet containing 15% fat, 1% cholesterol, and 0.5% cholate (ATHERO) for 12 weeks.
lesion formation, ABCG1*+/+ and ABCG1*−/− mice were fed an atherogenic diet for 12 weeks. As a result of the atherogenic diet, total serum cholesterol levels in both the control and experimental groups increased approximately 3-fold to values of 210±61 and 220±46 mg/dL, respectively. Also under these conditions no differences were observed between the ABCG1*−/− and ABCG1*+/+ mice.

The distribution of cholesterol among serum lipoproteins was analyzed by liquid chromatography. On the chow diet, ABCG1*−/− and ABCG1*+/+ mice mainly transport their cholesterol in the HDL fraction. Feeding the atherogenic diet led to a dramatic rise in both VLDL and LDL cholesterol levels, but no significant change in HDL cholesterol was observed after 12 weeks on the atherogenic diet (Figure 1A). The lipoprotein profiles of the two groups were essentially identical on both the standard chow diet and the atherogenic diet, indicating that ABCG1 deficiency did not affect serum lipid levels or the lipid distribution.

Deletion of ABCG1 has recently been suggested to lead to increased secretion of apoE by macrophages and elevated plasma apoE levels.14 To investigate the effect of ABCG1 deletion on serum apoE levels immunoblotting for apoE protein in the serum of mice fed the atherogenic diet for 12 weeks was performed. No significant difference was observed in serum apoE levels between ABCG1*−/− and ABCG1*+/+ mice (Figure 1B).

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

To investigate the importance of ABCG1 for atherosclerotic lesion development, we analyzed the aortic root and arch of ABCG1*+/+ and ABCG1*−/− mice after 12 weeks of atherogenic diet feeding (Figure 2). Representative photomicrographs of the aortic root of control mice and the mice deficient for ABCG1 are shown in Figure 2A. After 12 weeks on the atherogenic diet, a significant 1.9-fold increase in atherosclerotic lesion size was observed in the aortic root of ABCG1*−/− mice compared with ABCG1*+/+ littermates (24±7x103 μm² [n=6] and 46±6x103 μm² [n=7] for ABCG1*+/+ and ABCG1*−/− mice, respectively); Student t test P=0.034, and Mann–Whitney test P=0.050; Figure 2B). No atherosclerosis was observed in the aortic arch of both groups of animals (data not shown).

**ABCG1 Disruption and Lipid Homeostasis in Other Tissues**

Visual examination of the lungs of ABCG1*−/− mice showed abnormal lung morphology compared with controls. Macroscopic analysis of the lungs after perfusion revealed significant whitening of the lungs in ABCG1*−/− mice but not wild-type mice (Figure 3A). A detailed microscopic view of the lungs showed accumulation of large amounts of lipids in the subpleural macrophage-rich regions of the lungs in ABCG1*−/− mice, whereas no lipid accumulation was observed in the same regions in control animals on 12 weeks of atherogenic diet feeding.
Discussion

The availability of ABCG1−/− mice provides an important tool to study the function of the ABCG1 protein. Kennedy et al and Baldán et al demonstrated that in chow-fed ABCG1−/− mice extensive subpleural cellular macrophage lipid accumulation occurs in the lungs at the age of 6 months. Furthermore, after 9 weeks of feeding a high fat/high cholesterol diet containing 21% fat and 1.25% cholesterol already a 2-fold increase in lipids in the lung was observed. We observed in the ABCG1−/− mice on a diet with 15% fat, 1% cholesterol and 0.5% cholate for 12 weeks a marked change in lung morphology attributable to excessive accumulation of lipids in macrophages localized to the subpleural region. Baldán et al demonstrated that macrophages from ABCG1−/− mice accumulate cholesterol ester droplets when incubated with surfactant and that macrophage ABCG1 plays an essential role in pulmonary lipid homeostasis, consistent with the tissue-specific phenotype. In addition to the lung, we also

Figure 2. ABCG1 deficiency induces atherosclerotic lesion development. Atherosclerotic lesion development was analyzed at the aortic root of ABCG1+/+ and ABCG1−/− mice after feeding an atherogenic diet for 12 weeks. A, Representative photomicrographs of oil red O–stained lesions in the aortic root (magnification: 20×). B, Quantification of lesion development in the aortic root of ABCG1+/+ and ABCG1−/− mice. Values represent the means±SEM of 6 to 7 mice. *Statistical significant difference (P=0.034 and P=0.050) as compared with ABCG1+/+ mice (Student’s test and Mann–Whitney test, respectively).

Figure 3. ABCG1−/− mice show abnormal lung morphology compared with control ABCG1+/+ littermates. A, Representative picture of lungs isolated from two ABCG1+/+ (left) and ABCG1−/− (right) mice after 12 weeks of atherogenic diet feeding shows lipid deposition in white. B, Representative photomicrographs of oil red O–stained lung sections from ABCG1-deficient (ABCG1−/−) mice and wild-type littermate controls (ABCG1+/+) at different magnifications (5× and 20×) show extreme lipid loading in subpleural regions of the lung.

Figure 4. ABCG1 deficiency induces lipid loading in the spleen. Representative photomicrographs of oil red O–stained spleen sections from ABCG1-deficient (ABCG1−/−) mice and wild-type littermate controls (ABCG1+/+) at different magnifications (10× and 20×). Excessive lipid loading both within and surrounding the germinal center of the spleen can be observed in ABCG1−/− mice.
observed lipid accumulation in the spleen as well as a 1.9-fold increase in lesion formation in the arterial wall of ABCG1−/− mice. These latter phenomena may be best explained by the established ability of ABCG1 to facilitate cholesterol efflux from macrophages to HDL.5,6 However, because the ABCG1-deficient mice were on a C57Bl/6J genetic background it was obligatory to use cholesteryl plus cholate to induce atherosclerotic lesions. DNA microarray studies have indicated that cholesterol is required to induce in the liver genes involved in acute inflammation, whereas cholate induces expression of genes involved in extracellular matrix deposition and the accumulation of collagen.17 Both the cholesterol and cholate components of the atherogenic diet have distinct proatherogenic effects on gene expression.17 Cholate also facilitates cholesterol absorption and thus will increase cholesterol loading and hence hypercholesterolemia.18–20 Although we used this cholate-containing diet for a limited time (12 weeks), the possibility thus exists that an antiinflammatory effect of ABCG1 also contributes to the observed cardioprotective effect.

Cholesterol efflux from macrophages is a complex process relying on ABCA1, ABCG1, apoE, SR-BI, 27-hydroxylation of cholesterol, and aqueous diffusion.5,6,21–23 Interestingly, in studies using mice with combined deficiency of both LXRα and LXRβ (LXRα−/−β−/−) significant foam cell accumulation was observed in lung, spleen, and the arterial wall.24 Among the target genes for the LXRs are ABCA1, apoE, and ABCG1.25–27 Therefore, it was suggested that formation of cholesterol-laden macrophages owing to LXR deficiency results from inability to upregulate the ABC-transporter and ApoE-mediated cholesterol efflux from macrophages, the initial step in reverse cholesterol transport. It is striking to notice that LXRα−/−β−/− mice, although followed for 18 months do only develop initial lesions consisting of foam cells which do not progress into advanced atheromas.24 We also observed in our ABCG1-deficient mice that the deficiency in ABCG1-mediated cholesterol efflux did not cause advanced atherosclerosis but only increased initial lesions mainly consisting of lipid-laden macrophages. The main aim of the present study was the further assessment of the role of ABCG1 in atherosclerotic lesion formation, especially in relation to the apparently conflicting data of the effect of macrophage ABCG1 in LDL receptor–deficient mice.12–14 Both Baldán et al13 and Ranalletta et al14 (only at 11 weeks) observed a decrease in atherosclerosis in mice transplanted with ABCG1−/− bone marrow which was explained either by increased apoptosis or increased ABCA1 expression and apoE secretion in the ABCG1−/− macrophages, while we observed a moderate increase in atherosclerotic lesion size.12 The induction of ABCA1 expression and apoE levels are obviously evoked by missing the major ABCG1 function, which is cholesterol release from macrophages. The magnitude of macrophage cholesterol loading and thus induction of ABCA1 and ApoE will be dependent on serum cholesterol levels. At the presently achieved cholesterol levels of 210 to 220 mg/dL, we did not observe any compensatory increase in apoE levels (Figure 1B). However, it might still be possible that ABCG1 deficiency is affecting other processes, like oxysterol accumulation, that can lead to overcompensation mediated by nuclear receptor activation. In the Baldán study serum cholesterol levels of 1040 mg/dL were reached by feeding a high-fat diet containing 21% fat and 1.25% cholesterol for 16 weeks leading to atherosclerotic lesion sizes in the sinus of 500 000 μm2 and 300 000 μm2 for ABCG1+/− and ABCG1−/− mice, respectively.12 In the Ranalletta study mice were fed a 21% fat diet with 0.2% cholesterol for 7 and 11 weeks, resulting in serum cholesterol levels of 800 to 900 mg/dL and 1100 mg/dL, respectively.14 Lesion sizes for ABCG1+/+ and ABCG1−/− mice in the Ranalletta study were 84 000 μm2 and 78 000 μm2 at 7 weeks and 238 000 μm2 and 100 000 μm2 at 11 weeks after diet feeding.14 In the Out study average cholesterol levels of 650 mg/dL were obtained by feeding a 15% fat and 0.25% cholesterol containing fat diet for 6 and 12 weeks, leading to lesion sizes for ABCG1+/+ and ABCG1−/− mice of 50 000 μm2 and 70 000 μm2 at 6 weeks and 125 000 μm2 and 170 000 μm2 at 12 weeks.12

By putting the ABCG1−/− mice on a diet with 15% fat, 1% cholesterol, and 0.5% cholate, we could induce the development of initial atherosclerotic lesions with serum cholesterol levels of 210 to 220 mg/dL. Indeed under these conditions we did observe a 1.9-fold increase in atherosclerotic lesion formation (lesion sizes are for ABCG1+/+ and ABCG1−/− mice respectively 24 000 μm2 and 46 000 μm2), establishing that ABCG1 indeed has a protective function in initial lesion formation. Whereas deletion of macrophage ABCG1 recently has been shown to lead to increased secretion of apoE by macrophages and elevated plasma apoE levels in LDL receptor deficient mice,14 we did not observe any differences in serum apoE levels in the total body ABCG1 deficient mice.

Although each study protocol in the afore mentioned studies is different, we found a highly significant correlation (P=0.005) when the fold increase in atherosclerotic lesion size of in ABCG1−/− mice as compared with ABCG1+/− mice is plotted against total serum cholesterol values whereby at about 900 mg/dL serum cholesterol a switch from ABCG1’s protective function to lesion promotion is noticed (Figure 5). We propose that at serum cholesterol concentrations below the level of 900 mg/dL, the effects of ABCG1 deficiency are a direct result of
disruption of the cholesterol efflux function of ABCG1, whereas above 900 mg/dL induction of other efflux mechanisms dominate because of absence of the major ABCG1 function induced by ABCG1 deficiency (see Figure 5). Thus, at later stages in lesion formation and under conditions of excessive lipid loading, attributable to ABCG1 deficiency, apoptosis and other efflux mechanisms may be induced, leading to overcompensation for the major function of ABCG1 which is cholesterol release from macrophages. However, it is also possible that ABCG1 is affecting other processes that can actually enhance atherogenesis at high cholesterol levels.

An important question remains what the relative importance of is ABCG1 for atherosclerotic lesion formation in the human situation. Human ABCG1, as originally identified by Schmitz and coworkers, is a monocyte-derived macrophage is upregulated by cholesterol loading and LXR receptor activation leads to an increase in ABCG1 protein expression. The antiatherosclerotic action of LXR agonists and their effect on reverse cholesterol transport may be caused by the LXR-mediated effects on macrophage ABCG1, provided that macrophage ABCG1 can be considered in the human situation as atheroprotective. According to American Heart Association standards, total cholesterol levels in humans are considered “healthy” below 200 mg/dL. In present work, ABcg1 deletion disrupts lipid homeostasis in alveolar macrophages and moderately influences atherosclerotic lesion development in LDL receptor-deficient mice. Arterioscler Thromb Vasc Biol. 2006;26:2295–2300.


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