Expression of Human OSBP-Related Protein 1L in Macrophages Enhances Atherosclerotic Lesion Development in LDL Receptor–Deficient Mice

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Objective—The purpose of this study was to assess the role of macrophage OSBP-related protein 1L (ORP1L) in the development of atherosclerosis.

Methods and Results—C57BL/6 mice overexpressing human ORP1L in macrophages driven by scavenger receptor A promoter were generated. Bone marrow (BM) of the mice was transplanted into LDL receptor–deficient animals, and aortic root lesion area in the recipients was determined after Western-type diet feeding. The recipients of ORP1L BM displayed 2.1-fold increase (P<0.001) in lesion size as compared with recipients of wild-type littermate BM. Macrophages of the ORP1L BM recipients showed a decrease in ABCG1 and APOE mRNAs and proteins, and an increase in PLTP message; also the plasma PLTP activity was elevated. The effect of ORP1L on cholesterol efflux was assessed using macrophages loaded with [3H]cholesterol oleate-acLDL or labeled with [3H]cholesterol. The ORP1L transgenic macrophages displayed 30% reduction (P<0.01) in cholesterol efflux to HDL2, but not to apoA-I. ORP1L was shown to bind 25- and 22(R)-hydroxycholesterol, identifying it as an oxysterol binding protein. Furthermore, ORP1L attenuated the response of ABCG1 mRNA to 22(R)-hydroxycholesterol, the effect on ABCA1 being less pronounced.

Conclusions—The results demonstrate that macrophage ORP1L can act as a modulator of atherosclerotic lesion development and provide clues to the underlying mechanism. (Arterioscler Thromb Vasc Biol. 2007;27:1618-1624.)

Key Words: ABCG1 • apolipoprotein E • atherosclerosis • cholesterol efflux • macrophage • ORP1L • oxysterol binding protein • PLTP

Lipid-loaded monocyte-derived macrophage foam cells are characteristic constituents of the early atherosclerotic lesion, the fatty streak, and are present at all stages of lesion development. Macrophages also play a central role in the inflammatory signaling within the plaque, whereas hydrolytic enzymes secreted by macrophages and other inflammatory cell types have been suggested to influence plaque structure and stability. In addition to cholesterol, several oxysterols, 27-carbon oxygenated derivatives of cholesterol, are enriched in the atherosclerotic plaques in both humans and in animal models. Oxysterols have cytotoxic, proapoptotic, and proinflammatory effects and facilitate monocyte differentiation into macrophages. They have therefore been suggested to adversely affect lesion development and stability. However, certain oxysterols act as agonists of liver X receptors, transcription factors that facilitate cholesterol efflux from macrophages and removal from the circulation via the reverse cholesterol transport process, thus having beneficial properties in terms of atherosclerosis.

Oxysterol binding protein (OSBP), a cytosolic receptor for oxysterols, was isolated and molecularly cloned in the 1980s. Families of proteins (OSBP-related proteins (ORPs)) displaying sequence homology to the carboxy-terminal sterol binding domain of OSBP are present in practically all eukaryotic organisms. The mammalian ORP have been implicated as sterol sensors that regulate cellular functions ranging from sterol and neutral lipid metabolism to vesicle transport and cell signaling. However, until now, their role in the development of atherosclerosis has not been assessed.

We have previously shown that one of the human ORP proteins, ORP1L, is expressed in macrophages and, moreover, is upregulated on the differentiation of human monocytes into macrophages. Observations suggesting that ORP1L may play a role in atherogenesis. In the present study we generated transgenic C57BL/6 mice that overexpress ORP1L in macrophages, driven by scavenger receptor A promoter. Using the bone marrow transplantation technique,
we examined the effect of macrophage expression of human ORP1L on the size of aortic root lesions in LDLr<sup>−/−</sup> animals that were fed a Western-type diet. The results demonstrated a marked increase of lesion size in recipients of ORP1L transgenic bone marrow as compared with recipients of wild-type littermate bone marrow.

Materials and Methods

Antibodies and Other Reagents

The reagents used are specified in the supplemental materials (available online at http://atvb.ahajournals.org).

Generation of the Transgenic Mice

Transgenic C57B/6J mice carrying the human ORP1L cDNA under control of the macrophage-specific SR-A promoter were generated as described (see supplemental materials).

Bone Marrow Transplantation

Bone marrow from ORP1L transgenic mice or gene-negative littermate controls was transplanted into homozygous LDL receptor knockout (LDLr<sup>−/−</sup>) mice as described (see supplemental materials).

Histological Analysis of the Aortic Root

To analyze the development of atherosclerotic lesions at the aortic root, transplanted LDLr<sup>−/−</sup> mice were euthanized after 9 weeks on a Western-type diet. The area of aortic root atherosclerotic lesions was analyzed as described (see supplemental materials).

Preparation and acLDL-Loading of Human Primary Macrophages

Human primary macrophages were differentiated from buffy coat mononuclear cells using granulocyte-macrophage colony stimulating factor (GM-CSF), and loaded with acLDL as described (see supplemental materials).

Analysis of Plasma Lipids and Lipoproteins

Plasma lipid and lipoprotein analyses were performed as described (see supplemental materials).

mRNA Quantification

Total RNA was isolated from peritoneal or RAW264.7 cells and selected mRNAs quantified by real-time RT-PCR as described (see supplemental materials). The gene-specific primers used are listed in supplemental Table I.

Determination of Plasma PLTP Activity

PLTP activity was determined as previously described.<sup>14</sup>

Analysis of Macrophage Cholesterol Efflux

Peritoneal cells from 20-week-old male ORP1L TG mice or age-matched wild-type C57BL/6J animals were loaded with 24 hours with [3H]cholesterol oleate-labeled acLDL or labeled for 24 hours with [3H]cholesterol (see supplemental materials). Efflux of the radioactive cholesterol to human apoA-I or human HDL<sub>2</sub> was determined as described in the supplemental materials.

In Vitro Assay for Oxysterol Binding by ORP1L

The oxysterol binding capacity of ORP1L in the cytosol of transfected HEK293 cells was determined as described (see supplemental materials).

Preparation of Recombinant Adenoviruses and Transduction of RAW264.7 Cells

Recombinant adenoviruses expressing GFP (AdGFP) or ORP1L (AdORP1L) were generated and used for transduction of RAW264.7 cells as described (see supplemental materials).

Results

The ORP1L Transgenic Animals

To generate transgenic (TG) mice that selectively express human ORP1L in macrophages, we used an expression construct in which the ORP1L cDNA is under control of the scavenger receptor A (SR-A) promoter. Expression of the transgene in the peritoneal macrophages of the gene-positive animals was verified by RT-PCR with primers specific for the human ORP1L mRNA (data not shown) and by Western blotting with ORP1L antibodies. For the present study we selected transgenic F0 progeny which, assuming that the antibodies used recognize equally the mouse and human proteins, express human ORP1L at levels 15-fold higher than the endogenous mouse ORP1L level (Figure 1A). The expression of the transgenic ORP1L was compared with that in primary human monocyte-macrophages before and after loading with acLDL (Figure 1B). The acLDL loading increased ORP1L protein in the human macrophages by 75% to 80%. The ORP1L level in the TG mouse peritoneal macrophages was similar to that in the acLDL-loaded human cells. The endogenous ORP1L level in mouse peritoneal macrophages appeared markedly lower than that in the in vitro differentiated human macrophages.

Macrophage ORP1L Enhances Atherosclerotic Lesion Development in LDLr<sup>−/−</sup> Mice

To assess the role of macrophage ORP1L in atherosclerotic lesion development, we used bone marrow (BM) transplantation to selectively express human ORP1L in hematopoietic cells. BM from transgenic C57BL/6 mice that express human ORP1L in macrophages was transplanted into LDLr<sup>−/−</sup> mice (ORP1L → LDLr<sup>−/−</sup>), which represent an established model for the development of atherosclerosis. LDLr<sup>−/−</sup> mice transplanted with BM from nontransgenic control littermates were used as controls (WT → LDLr<sup>−/−</sup>). Successful reconstitution

Figure 1. A, Overexpression of human ORP1L in peritoneal macrophages of the transgenic mice. Peritoneal cell proteins (15 μg/lane) from gene-negative littermates (WT) or ORP1L transgenic animals (TG) were resolved on SDS-PAGE and Western blotted using antibodies against ORP1L or β-actin (indicated on the right). Data for 2 animals from each group is shown. B, Comparison of the transgene expression level with endogenous ORP1L in primary human monocyte-macrophages. Equal amounts of protein (15 μg/lane) were loaded. a, human macrophages; b, human macrophages loaded for 48 hours with acLDL at 25 μg protein/mL; c, ORP1L transgenic macrophages. Two parallel specimens are shown. Mobilities of molecular mass markers are indicated on the left.
of the transplanted bone marrow was verified by PCR with human ORP1L-specific primers using genomic DNA from the bone marrow of WT → LDLr−/− or ORP1L → LDLr−/− animals (supplemental Figure I). To induce atherosclerotic lesion development, the transplanted mice were allowed to recover for 8 weeks and then fed a Western-type diet for 9 weeks, followed by analysis of atherosclerotic lesion size in the aortic root. Expression of human ORP1L in macrophages induced a 2.1-fold increase in lesion size [271±23×10^3 μm^2 in ORP1L → LDLr−/− mice (n=19) versus 130±23×10^3 μm^2 in WT → LDLr−/− mice (n=17, P<0.001; Figure 2A)]. The macrophage content of the lesions of ORP1L → LDLr−/− mice was 51±4% and the collagen content 2.3±0.5% (Figure 2B and 2C). These values did not differ significantly from those determined for the WT → LDLr−/− mice (46±4% and 1.9±0.9%, respectively). No difference was observed in the plasma total or free cholesterol, triglyceride, or choline-containing phospholipid concentrations between the ORP1L → LDLr−/− and WT → LDLr−/− mice before or after the Western-type diet feeding (supplemental Table II), nor in the plasma lipoprotein profiles of the animals (data not shown).

ORP1L Downregulates ABCG1 and ApoE and Induces PLTP
To investigate the mechanisms underlying the increase of lesion size in recipients of ORP1L BM, we quantified the mRNAs for 12 genes involved in macrophage lipid metabolism or inflammatory signaling, in peritoneal macrophages of the BM recipients. The mRNAs for ABCG1, ABCG5, and APOE were significantly downregulated in the cells from ORP1L → LDLr−/− mice as compared with WT → LDLr−/− controls (supplemental Figure II). Moreover, the PLTP and interleukin-1β (IL-1β) mRNAs were induced. To verify the downregulation of ABCG1 and apoE at the protein level, the peritoneal cells were analyzed by Western blotting. A 54% decrease in ABCG1 and 68% decrease in apoE in the ORP1L → LDLr−/− mice, as compared with controls, was observed (Figure 3A through 3C). Determination of plasma PLTP activity revealed a 30% increase in the ORP1L → LDLr−/− animals as compared with controls (22.92±1.65 μmol/mL/h versus 17.61±0.72 μmol/mL/h; P<0.05).

Macrophase ORP1L Impairs Cholesterol Efflux to Spherical High-Density Lipoproteins (HDL)
The downregulation of ABCG1 and apoE, which participate in cellular cholesterol efflux,15–17 led us to investigate whether ORP1L overexpression affects macrophase cholesterol efflux. Peritoneal macrophages from ORP1L TG and age-matched control C57BL/6 mice were loaded with [3H]cholesterol olate-labeled acLDL or with free [3H]cholesterol, and the efflux of [3H]cholesterol to apoA-I or HDL2 was measured. Independent of the labeling method, the efflux from ORP1L TG macrophages to HDL2 was significantly reduced (acLDL, by 31%, P<0.01; free cholesterol, by 28%, P<0.01) as compared with controls, whereas no difference was observed in cholesterol efflux to apoA-I (Figure 3D and 3E).

ORP1L Is an Oxysterol Binding Protein
To study the mechanisms underlying the ORP1L effects, we investigated whether the protein is able to bind oxysterols. HEK293 cells were transfected with the human ORP1L cDNA, the rabbit OSBP cDNA (as a reference), or the plain vector. Thereafter, cytosolic extracts of the cells were assayed for [3H]25OHC or [3H]22(R)OHC binding capacity. ORP1L-containing cytosol showed similar 25OHC binding activity as OSBP (Figure 4). The ORP1L signal was competed 65% to 80% by a 40-fold excess of unlabeled 25OHC and was markedly higher than background 25OHC binding in lysate...
of mock-transfected cells. With [3H]22(R)OHC, an established ligand of LXR, ORP1L displayed a stronger specific signal than with 25OHC, whereas OSBP failed to bind this oxysterol above background levels.

**ORP1L Modulates the Response of ABCA1 and ABCG1 mRNAs to 22(R)OHC**

To test the hypothesis that ORP1L could modulate the response of LXR target genes to oxysterols, we treated adenovirally transfected mouse RAW264.7 macrophages for 6 hours with increment concentrations of 22(R)OHC, and quantified the ABCG1 and ABCA1 mRNA levels in the cells (Figure 5). The baseline level of ABCG1 but not ABCA1 mRNA was in AdORP1L-infected cells significantly reduced (by 53%; \( P < 0.01 \)), similar to the macrophages of ORP1L BM recipient mice. Although both mRNAs displayed a dose-dependent induction in cells infected with the control virus (AdGFP), the response of the ABCG1 message to 0.625 \( \mu \)mol/L 22(R)OHC was markedly attenuated in AdORP1L-transduced cells (relative difference to AdGFP-transduced cells 66%, \( P < 0.001 \); Figure 5A). The difference was not significant at the higher concentrations of the oxysterol.

**Figure 3.** ORP1L overexpression results in the reduction of macrophage apoE and ABCG1 proteins and of cholesterol efflux to HDL.

Peritoneal cell proteins (15 \( \mu \)g/lane) from WT \( \rightarrow \) LDLr\(^{-/-} \) (WT) or ORP1L \( \rightarrow \) LDLr\(^{-/-} \) (TG) mice were resolved on SDS-PAGE and Western blotted using antibodies against ABCG1, apoE, or \( \beta \)-actin (A). Data for 4 animals from each group are shown. Quantification of the Western blot signals for ABCG1 (B) and apoE (C), normalized using \( \beta \)-actin signal. The data represents mean±SEM (n=4; \( * P < 0.05, **P < 0.01 \)). D and E, Peritoneal cells from wild-type (WT) and ORP1L transgenic (TG) mice were loaded with [3H]cholesterol oleate labeled acLDL (D) or with [3H]cholesterol (E). Efflux of the [3H]cholesterol to human apoA-I (10 \( \mu \)g/mL) or HDL\(_2\) (25 \( \mu \)g/mL) during 4 hours was determined. NA, no acceptor. The data represents mean±SEM (n=3 to 6, \( **P < 0.01 \)).
showed an increase of the peritoneal cells of the mice. Moreover, the fact that the peritoneal cells of the ORP1L bone marrow recipients showed an increase of IL-1β mRNA expression indicates that also inflammatory signaling by the transgenic macrophages could be affected.

ABCG1 has a critical role in cholesterol efflux to spherical HDL and thereby prevents lipid accumulation in hepatocytes and macrophages.26–28 Therefore, we find it likely that the observed impairment of macrophage cholesterol efflux to spherical HDL is at least in part attributable to reduced ABCG1 expression. However, bone marrow transplantation studies addressing the effects of macrophage ABCG1 deficiency on atherogenesis have yielded controversial results.21–23 Moreover, crossing of ABCG1 transgenic mice with LDLr<sup>−/−</sup> animals revealed increased atherosclerosis attributable to ABCG1 overexpression in the liver and macrophages, despite enhanced cholesterol efflux from macrophages.24 Therefore, it is difficult to firmly define the functional consequences of the reduced macrophage ABCG1 expression observed in the present study.

ApoE plays a major antiatherogenic role by facilitating the clearance of chylomicron remnants from circulation via LDL-receptor related protein. It is also associated with subpopulations of HDL, can act as an activator of lecithin-cholesterol acyltransferase (LCAT), and mediates the hepatic uptake of HDL via LDL receptors, functions important for the reverse cholesterol transport process.15 Furthermore, apoE-containing large HDL particles act as acceptors for macrophage cholesterol efflux, especially in cholesterol ester transfer protein (CETP)–deficient animal models and human subjects.16,25 Bone marrow transplantation studies addressing the role of macrophage apoE show that expression of human apoE in macrophages of apoE-deficient mice is atheroprotective,29 whereas transplantation of apoE-deficient macrophage stem cells into wild-type mice promotes atherosclerosis.27 Thus, the reduction of apoE may contribute to the increase of lesion size observed in the ORP1L BM recipients.

Figure 5. ORP1L modulates the response of ABCG1 mRNA to 22(R)OHC. RAW264.7 cells were transduced with a control adenovirus (AdGFP) or ORP1L recombinant virus (AdORP1L), and treated for 6 hours with increment concentrations of 22(R)OHC (indicated at the bottom). The ABCG1 (A) and ABCA1 (B) mRNAs were thereafter quantified. The results represent mean±SEM (n=4; *P<0.05, **P<0.01, ***P<0.001).

Figure 4. ORP1L binds oxysterols. Cytosolic extracts of HEK293 cells transfected with the plain vector (Mock), ORP1L cDNA, or rabbit OSBP cDNA were assayed for [3H]25OHC or [3H]22(R)OHC binding activity. The [3H]oxysterol concentrations used are identified at the bottom. Closed bars, no competition; Open bars, competition with a 40-fold excess of corresponding unlabeled oxysterol. Representative experiments performed in duplicate are shown.
The role of PLTP in the development of atherosclerotic lesions is far from clear. Systemic PLTP deficiency was reported to alleviate atherosclerosis via reduction of apolipoprotein B secretion, improvement of the anti-inflammatory properties of HDL, and increased protection of LDL from oxidation. However, bone marrow transplantation studies addressing the effect of macrophage PLTP deficiency on lesion development have yielded controversial results, whereas increased PLTP expression seems invariably proatherogenic. In the light of these investigations, it is plausible that the observed increase in macrophage PLTP expression and activity in plasma may contribute to the enhancement of lesion development in the ORP1L BM recipients.

We have previously shown that ORP1L interacts directly with the small GTPase Rab7 on late endosomes (LE) and modifies the subcellular distribution and membrane trafficking functions of the LE. The protein has been suggested to act as a sensor that relays signals from lipid cues to the late endocytic membrane trafficking machineries. Importantly, we now show that the protein also binds oxysterols. ORP1L is shown to bind both 25OHC and 22(R)OHC, whereas OSBP included as a reference failed to bind 22(R)OHC above background levels. This suggests that different ORPs may have distinct effects on cellular lipid metabolism attributable to differences in ligand specificity.

The ABGC1, ABGC5, PLTP, and APOE genes are subject to transcriptional regulation by the LXR. In addition to the control of cellular cholesterol efflux and the reverse cholesterol transport process, the LXR also display antiinflammatory activity by repressing, presumably via an indirect mechanism, a set of proinflammatory genes that include IL-1β and IL-6.

A tempting possibility is that excess ORP1L could interact with cellular oxysterols, resulting in altered expression of LXR targets. This hypothesis is corroborated by the finding that ORP1L modulated in RAW264.7 cells the response of the ABCG1 mRNA to the exogenously added LXR agonist 22(R)OHC. Why ORP1L had only a minor effect on ABCA1 remains to be investigated. On the other hand, disturbance of the normal endocytic pathway function by excess ORP1L could alter the intracellular trafficking of LDL-derived cholesterol and thus its conversion into oxysterols by sterol hydroxylases located in mitochondria and in the endoplasmic reticulum. However, the facts that ORP1L overexpression did not change ABCA1 mRNA abundance and that the PLTP message was elevated in ORP1L BM recipients suggest that the observed effects of ORP1L are complex and not attributable to a uniform stimulatory or inhibitory impact of ORP1L on the LXR.

We have previously shown that ORP1L overexpression in nonmacrophage cells also transfected with LXR and RXR plasmids, mildly enhanced an LXRE-dependent reporter in the presence of 22(R)OHC or a nonsterol LXR agonist. However, because of differences in the experimental systems used and in the content of individual oxysterol species present in different cell types, it is difficult to directly compare the reporter assay data and the present one.

A change in endocytic pathway function caused by ORP1L and a resulting disturbance of intracellular cholesterol trafficking could directly affect the availability of cholesterol for efflux from the plasma membrane. There is increasing evidence that manipulation of endocytic membrane trafficking, for instance by overexpression of small Rab GTPases, can alter cholesterol efflux from cells. However, the fact that ORP1L failed to enhance the transfer of cholesterol to apoA-I suggests that the availability of cholesterol for efflux in general was not altered, arguing for a specific effect mediated via distinct target proteins such as ABCG1.

The present study is the first investigation addressing the impact of the OSBP-related protein family on atherogenesis, and demonstrates that these proteins are promising targets for the development of drugs affecting the development of atherosclerotic lesions. Our work also provides clues to the mechanisms by which ORP1L induces an increase of plaque size, and motivates future work to elucidate the role of the entire ORP family in cardiovascular diseases.

Acknowledgments

We are grateful to Sari Nuutila, Seija Puomilahti, and Pirjo Ranta for skilled technical assistance, to Jari Metso and Mikko Muurola for help with lipid and lipoprotein analysis, and to Shuhei Nakanishi for preparing the primary human macrophages. The labeled acLDL was kindly provided by Riikka Wikstedt.

Sources of Funding

This work was supported by The Netherlands Organization of Scientific Research (grant 917.66.301 to M.v.E.), The Netherlands Heart Foundation (grant 2001T041 to M.v.E.), The Academy of Finland (grants 206298, 113013, 118720 to V.M.O.), The Sigrid Juselius Foundation (to M.J. and V.M.O.), The Finnish Foundation for Cardiovascular Research (to M.J. and V.M.O.), The Magnus Ehrnrooth Foundation (to C.E.), and The Finnish Society of Sciences and Letters (to C.E. and V.M.O.).

Disclosures

None.

References


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Arterioscler Thromb Vasc Biol. 2007;27:1618-1624; originally published online May 3, 2007; doi: 10.1161/ATVBAHA.107.144121
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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Supplementary Materials and Methods

Antibodies and other reagents
The rabbit antiserum against ORP1L has been described (Johansson et al., Mol Biol Cell. 2003;14:903-15). Rabbit antibodies against ABCG1 were from Novus Biologicals (Littleton, CO), anti-apoE from Biodesign International (Saco, ME), monoclonal anti-β-actin (clone JLA20) from the Developmental Studies Hybridoma Bank (Univ. of Iowa), and monoclonal Xpress tag antibody from Invitrogen (Carlsbad, CA). Lipid-free human apoA-I was kindly provided by Dr. Peter Lerch (Swiss Red Cross, Bern, Switzerland). HDL2 was purified from human plasma by ultracentrifugation (Havel et al., J Clin Invest. 1955;34:1345-53). [3H]25OHC was kindly provided by C. Thiele (Max-Planck-Institute of Molecular Cell Biology and Genetics, Dresden, Germany) and [3H]22(R)OHC was from American Radiolabeled Chemicals (St. Louis, MO). Unlabelled 25OHC and 22(R)OHC were from Sigma-Aldrich (St. Louis, MO).

Generation of ORP1L transgenic mice
The full-length human ORP1L cDNA (accession number AF323726) was inserted in the BamHI site of pAL1, which places it under control of the macrophage-specific human SR-A promoter (Horvai et al., Proc Natl Acad Sci U S A. 1995;92:5391-5). The expression cassette was removed by digestion with Xhol and NotI, and purified from agarose gels. The cassette was microinjected into the male pronuclei of fertilized oocytes of C57B/6JOLAhsd mice (Harlan Nederland, AD Horst, the Netherlands), which were transplanted into the oviducts of Hsd:ICR (CD-1) foster mothers. Among the progeny, ORP1L transgenic founders were screened by PCR using the gene-specific primers CGGAGCAACAGCTTCTCCATCAC (forward) and TCAAGCATGCTTCTGTCTTC (reverse). The seven gene-positive founders were mated with C57BL/6 animals to produce an N1 generation, and gene-positive N1 animals to produce an F0 generation. Selected F0 progeny with abundant, 5-6-fold overexpression of ORP1L in peritoneal macrophages as compared to the endogenous level (analysis by Western blotting), as well as gene-negative littermates, were used as bone marrow donors.
Bone marrow transplantation

Homozygous LDL receptor knockout (LDLr\(^{-/-}\); C57Bl/6 N5) mice were obtained from The Jackson Laboratory (Bar Harbor, ME, USA) as mating pairs and bred at the Gorlaeus Laboratory, Leiden, The Netherlands. Mice were housed in sterilised filter-top cages and given unlimited access to food and water. Mice were maintained on sterilised regular chow, containing 4.3% (w/w) fat and no cholesterol (RM3, Special Diet Services, Witham, UK), or were fed a semi-synthetic Western-type diet, containing 15% (w/w) fat and 0.25% (w/w) cholesterol (Diet W, Special Diet Services, Witham, UK). Drinking water was supplied with antibiotics (83 mg/L ciprofloxacin and 67 mg/L polymyxin B sulphate) 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. To induce bone marrow aplasia, female LDLr\(^{-/-}\) animals (age: 10 weeks) were exposed to a single dose of 9 Gy (0.19 Gy/min, 200 kV, 4 mA) X-ray total body irradiation, using an Andrex Smart 225 Röntgen source (YXLON International, Copenhagen, Denmark) with a 6-mm aluminium filter, one day before transplantation. Bone marrow was isolated by flushing the femurs and tibias from male ORP1L transgenic mice or wild-type littermates with phosphate-buffered saline. Single-cell suspensions were prepared by passing the cells through a 30-μm nylon gauze. Irradiated recipients received 0.5 x 10\(^7\) bone marrow cells by intravenous injection into the tail vein, followed by an 8-week recovery period on chow diet.

Histological analysis of the aortic root

To analyse the development of atherosclerosis at the aortic root, transplanted LDLr\(^{-/-}\) mice were sacrificed after 9 weeks on the Western-type 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 was excised and stored in 3.7% neutral-buffered formalin (Formal-fixx\(^{\circledR}\), Shandon Scientific Ltd., England). 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, England). Mean lesion area (in µm²) was calculated from 10 oil red O-stained sections, starting at the appearance of the tricuspid valves. For the assessment of the macrophage area sections were immunolabeled with MOMA-2 (Research Diagnostics Inc; dilution 1:50). The MOMA-2-positive lesion area was subsequently quantified using the Leica image analysis system. The amount of collagen in the lesions was determined using Masson’s Trichrome Accustain according to manufacturer’s instructions (Sigma Diagnostics). All quantifications were done blinded by computer-aided morphometric analysis using the Leica image analysis system.

**Preparation and acLDL-loading of human primary macrophages**

Human monocyte-derived macrophages were obtained by cell culturing from 60 mL of fasting blood containing citrate as an anticoagulant. Buffy coat was separated by centrifugation, diluted with PBS, layered over Ficoll-Paque PLUS (Amersham Biosciences AB, Uppsala, Sweden), and centrifuged for 30 min at 600 x g at +20 °C. The isolated mononuclear cells were washed three times with PBS to remove excess platelets and the cell pellet was suspended in DMEM (with 4.5 g/L glucose, with L-glutamine, without Na-pyruvate)(Cambex Bio Science, Verviers, Belgium) supplemented with penicillin/streptomycin. Cells were counted, plated on 24-well plates (1.5 x 10⁶ cells per well) and allowed to attach to the wells for 1 h. Next, the cells were washed three times with PBS and macrophage-SFM medium (Invitrogen, Carlsbad, CA) supplemented with GM-CSF (Nordic Biosite AB, Täby, Sweden). The medium was changed every 2 to 3 days. After seven days in culture the macrophages were washed twice with PBS and loaded for 48 h with 25 µg protein/well of acetyl-LDL in DMEM, penicillin/streptomycin.

**Analysis of plasma lipids and lipoproteins**

Total plasma cholesterol (Kit 1489232, Roche Diagnostics GmbH, Mannheim, Germany), choline-containing phospholipids (Kit 990-54009, Wako Chemicals GmbH, Neuss, Germany) and triglycerides (Kit 1488872, Roche Diagnostics) were measured using fully enzymatic methods. Serum lipoproteins were fractionated (pools from 6-7 mice, vol. 200 µl) by fast-performance liquid chromatography (FPLC) using a Superose
6HR 10/30 size-exclusion chromatography column (GE Healthcare, Buckinghamshire, UK).

**Western blotting**
Protein specimens for SDS-PAGE were prepared by homogenizing peritoneal cells or human monocyte-macrophages in 250 mM Tris-HCl, pH 6.8, 8% SDS, protease inhibitor cocktail (Roche Diagnostics, Mannheim, Germany). The crude extracts were cleared by centrifugation at 16,000 x g for 3 min, and protein concentration of the supernatant determined by the Dc assay (BioRad, Hercules, CA). Equal amounts of the proteins were electrophoresed and Western blotted using the primary antibodies specified above. The bound antibodies were visualized by using horseradish peroxidase-conjugated goat anti-mouse or anti-rabbit IgG (Bio-rad, Hercules, CA) and enhanced chemiluminescence (ECL; GE Healthcare).

**Quantification of peritoneal macrophage mRNAs**
Total RNA was isolated from peritoneal cells with the RNeasy Mini kit (Qiagen, Valencia, CA) according to the manufacturer’s instructions. The RNA (2 µg) was treated with DNase I (Promega, Madison, WI) in the presence of RNase Inhibitor (Promega) and reverse-transcribed by using Superscript II (Invitrogen) and random hexamer primers (Applied Biosystems, Foster City, CA). Each RNA sample was amplified in triplicate for the genes of interest and the housekeeping marker 36B4, on a 7000 Sequence Detection System (Applied Biosystems) by using SYBR-green (ABgene, Surrey, UK). Sequences of the primers used are listed in Table I (see www.ahajournals.org). The threshold was set in the linear range of fluorescence, and a threshold cycle (Ct) was measured for each well. The data was analyzed as described previously (Pfaffl, *Nucleic Acids Res.* 2001; 29: e45).

**Analysis of macrophage cholesterol efflux.** Peritoneal cells isolated from 20-week old male *ORP1L* TG mice or age-matched wild-type C57BL/6 animals were plated on 24-well plates at 1 x 10^6 cells/well in Dulbecco’s Modified Eagle’s Medium (DMEM; Sigma-Aldrich, St. Louis, MO) supplemented with 20% fetal calf serum (FCS), 100 U/ml penicillin, and 100 µg/ml streptomycin. The macrophages attached after a 6-h incubation
at 5% CO₂, 37°C, were loaded for 24 h with [³H]cholesterol oleate labelled acLDL (20 µg protein, and 750,000 dpm per well) prepared as described in (Lee-Rueckert et al., J Lipid Res. 2006;47:1725-32) or labelled for 24 h with [³H]cholesterol (0.5 µCi per well) in the presence of an ACAT inhibitor (2 µg/ml PKF 058-035; Novartis, Basel, Switzerland). After three washes and a 2-h incubation in serum-free medium containing 0.2% bovine serum albumin, the cells were incubated for 4 h with human apoA-I (10 µg/ml) or human HDL₂ (25 µg protein/ml) in serum-free medium. The efflux media were removed and the cells dissolved in 0.2M NaOH. Aliquots of both the cells and the medium were analyzed by liquid scintillation counting, and the cellular protein was determined as described above.

There was no significant difference in the efficiency of acLDL loading between the control (4832 ± 132 dpm/µg cell protein) and ORP1L TG (4906 ± 116 dpm/µg cell protein) macrophages. Moreover, the distribution of cellular [³H]cholesterol between free and esterified forms after the acLDL loading was determined by thin-layer chromatography, and did not differ between ORP1L transgenic and control macrophages (proportion of free cholesterol 11.0 ± 0.8 % or 11.6 ± 0.7%, respectively).

In vitro assay for oxysterol binding by ORP1L

HEK293 cells cultured on 6 cm dishes in Eagle’s minimal essential medium with Earle’s salts (EMEM, Sigma-Aldrich) supplemented with 10% FCS and non-essential amino acids, were transfected with Xpress epitope-tagged human ORP1L or rabbit OSBP cDNAs in pcDNA4HisMaxC (Invitrogen). Binding of [³H]25OHC (kindly provided by Dr. C. Thiele, Max-Planck-Institute of Molecular Cell Biology and Genetics, Dresden, Germany) to OSBP (positive control) or ORP1L in cytosolic extracts of the transfected cells was assayed as described previously (Taylor and Kandutsch, Methods Enzymol. 1985;110:9-19). Extract of cells transfected with the plain vector was used as a negative control. Briefly, cytosolic fractions were incubated overnight with 5, 10, 20, and 40 nM [³H]25OHC or [³H]22ROHC in the absence or presence of a 40-fold excess of corresponding unlabelled oxysterol. The free sterol was thereafter removed with charcoal-dextran, and the protein-bound [³H]sterol remaining in the supernatant was
analyzed by liquid scintillation counting. The data was normalized for protein quantity based on Western blotting of aliquots of the extracts using Xpress antibody.

**Preparation of recombinant adenoviruses and transduction of RAW264.7 cells**

Recombinant adenoviruses expressing GFP (AdGFP) or ORP1L (AdORP1L) were generated using pAdenovator-CMV5-IRES-GFP (QbioGene, Illkirch, France), and the AdEasy system as described (Yan et al., *Arterioscler Thromb Vasc Biol.* 2007; Epub Febr 15). RAW264.7 mouse macrophages were cultured on 6-well plates in Dulbecco’s Modified Eagle’s Medium (DMEM; Sigma-Aldrich, St. Louis, MO), 10% FCS, with penicillin/streptomycin, and transduced with recombinant adenoviruses at a multiplicity of 100 p.f.u. per cell for 20 h in medium supplemented with 5% lipoprotein-deficient FCS. For the last 6 h, increment concentrations of 22(R)OHC were added in the wells, after which total RNA was isolated and the *ABCA1* and *ABCG1* mRNAs quantified as above.
Table I. Oligonucleotide primers used for mRNA quantification by real-time RT-PCR

<table>
<thead>
<tr>
<th>mRNA</th>
<th>Forward primer 5’-3’</th>
<th>Reverse primer 5’-3’</th>
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</thead>
<tbody>
<tr>
<td>IL-1β</td>
<td>caa cca aca agt gat att tct cat g</td>
<td>gat cca cac tct cca gct gca</td>
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<tr>
<td>IL-6</td>
<td>ctt cag gag act ttc atc cag tt</td>
<td>gaa gta ggg aag ggc gtt g</td>
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<td>IL-10</td>
<td>ggt tgc caa agc ctt atc gga</td>
<td>acc tgc tcc act gcc tgt ct</td>
</tr>
<tr>
<td>TNFα</td>
<td>ttc tgt cta ctt aac ttc ggg gtt atc cgg tcc</td>
<td>gta tga gaty agc aaa tcc gct gac ggt gtg tgg g</td>
</tr>
<tr>
<td>ABCA1</td>
<td>cgt ttc cgg gaa gtt tcc ta</td>
<td>gct aga gat gag aag gag gat gga</td>
</tr>
<tr>
<td>ABCG1</td>
<td>cca gac cct ttt tga aag gga tct c</td>
<td>gcc aga ata ttc atg agt gtt gac</td>
</tr>
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<td>ABCG5</td>
<td>tggatcacaaccctatgctaa</td>
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</tr>
<tr>
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<td>cgcaagtagtgatcctcga</td>
</tr>
<tr>
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<td>gga gat gaa ggc agg aca gaa g</td>
</tr>
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<td>aac gcc att gcc tgg aag gaa</td>
</tr>
<tr>
<td>SR-B1</td>
<td>tgt gcc tgt ttt tgt gga tg</td>
<td>gga ttc ggg tgt cat gaa gg</td>
</tr>
<tr>
<td>MMP-9</td>
<td>agc aca aca gct gac tac gat aag</td>
<td>ggc ctt cgg gca cgc tgg aat gat cta a</td>
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<tr>
<td>36B4</td>
<td>cat gct caa ctt ccc ctt ctc c</td>
<td>ggg aag gtt taa tcc gtc tcc aca g</td>
</tr>
</tbody>
</table>

The abbreviations are: IL, interleukin; TNF, tumor necrosis factor; ABC, ATP-binding cassette transporter; PLTP, phospholipid transfer protein; APOE, apolipoprotein E; SR-B1, scavenger receptor class B type 1; MMP, matrix metalloproteinase; 36B4, acidic ribosomal phosphoprotein 36B4
Table II. Plasma lipid values of the WT→LDLr<sup>−/−</sup> and ORP1L→LDLr<sup>−/−</sup> mice

<table>
<thead>
<tr>
<th></th>
<th>TC&lt;sup&gt;1&lt;/sup&gt; (mmol/L)</th>
<th>FC&lt;sup&gt;2&lt;/sup&gt; (mmol/L)</th>
<th>TG&lt;sup&gt;3&lt;/sup&gt; (mmol/L)</th>
<th>PL&lt;sup&gt;4&lt;/sup&gt; (mmol/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT→LDLr&lt;sup&gt;−/−&lt;/sup&gt;</td>
<td>B&lt;sup&gt;5&lt;/sup&gt; 7.60 ± 0.23</td>
<td>2.00 ± 0.07</td>
<td>2.01 ± 0.20</td>
<td>4.84 ± 0.12</td>
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<tr>
<td></td>
<td>A&lt;sup&gt;6&lt;/sup&gt; 21.97 ± 1.34</td>
<td>6.21 ± 0.38</td>
<td>2.99 ± 0.28</td>
<td>7.91 ± 0.25</td>
</tr>
<tr>
<td>ORP1L→LDLr&lt;sup&gt;−/−&lt;/sup&gt;</td>
<td>B 7.46 ± 0.27</td>
<td>1.95 ± 0.07</td>
<td>1.66 ± 0.10</td>
<td>5.07 ± 0.11</td>
</tr>
<tr>
<td></td>
<td>A 24.22 ± 1.38</td>
<td>6.82 ± 0.45</td>
<td>3.06 ± 0.34</td>
<td>8.31 ± 0.32</td>
</tr>
</tbody>
</table>

<sup>1</sup>Total cholesterol;<sup>2</sup>Free Cholesterol;<sup>3</sup>Triglyceride;<sup>4</sup>Choline-containing phospholipids
<sup>5</sup>Before and<sup>6</sup>after western-type diet
<sup>7</sup>The values represent mean ± s.e.m. (WT→LDLr<sup>−/−</sup>, n = 18; ORP1L→LDLr<sup>−/−</sup>, n = 19)
Figure I. Successful reconstitution of the transplanted bone marrow was verified by PCR with human ORP1L-specific primers using genomic DNA from the bone marrow of WT → LDLr−/− (WT) or ORP1L → LDLr−/− (TG) animals. Data for two animals from each group is shown.
Figure II. ORP1L overexpression results in down-regulation of macrophage ABCG1, ABCG5, and APOE mRNA expression and induction of the IL-1β and PLTP messages. Total RNA isolated from peritoneal cells of the WT → LDLr⁻/⁻ (WT) or ORP1L → LDLr⁻/⁻ (TG) mice was subjected to real-time RT-PCR analysis of selected endogenous mRNAs (identified at the bottom). The data represents relative mRNA quantity, mean ± s.e.m. (n = 6, *p<0.05, **p<0.01).