Reduced ABCA1-Mediated Cholesterol Efflux and Accelerated Atherosclerosis in Apolipoprotein E–Deficient Mice Lacking Macrophage-Derived ACAT1

Yan Ru Su, MD; Dwayne E. Dove, PhD; Amy S. Major, PhD; Alyssa H. Hasty, PhD; Branden Boone, MS; MacRae F. Linton, MD; Sergio Fazio, MD, PhD

Background—Macrophage acyl-coenzyme A:cholesterol acyltransferase 1 (ACAT1) and apolipoprotein E (apoE) have been implicated in regulating cellular cholesterol homeostasis and therefore play critical roles in foam cell formation. Deletion of either ACAT1 or apoE results in increased atherosclerosis in hyperlipidemic mice, possibly as a consequence of altered cholesterol processing. We have studied the effect of macrophage ACAT1 deletion on atherogenesis in apoE-deficient (apoE−/−) mice with or without the restoration of macrophage apoE.

Methods and Results—We used bone marrow transplantation to generate apoE−/− mice with macrophages of 4 genotypes: apoE+/+/ACAT1+/+ (wild type), apoE+/−/ACAT1−/− (ACAT−/−), apoE−/−/ACAT1+/+ (apoE−/−), and apoE−/−/ACAT1−/− (2KO). When macrophage apoE was present, plasma cholesterol levels normalized, and ACAT1 deficiency did not have significant effects on atherogenesis. However, when macrophage apoE was absent, ACAT1 deficiency increased atherosclerosis and apoptosis in the proximal aorta. Cholesterol efflux to apoA-I was significantly reduced (30% to 40%; P < 0.001) in ACAT1−/− peritoneal macrophages compared with ACAT1+/+ controls regardless of apoE expression. 2KO macrophages had a 3- to 4-fold increase in ABCA1 message levels but decreased ABCA1 protein levels relative to ACAT1+/+ macrophages. Microarray analyses of ACAT1−/− macrophages showed increases in proinflammatory and procollagen genes and decreases in genes regulating membrane integrity, protein biosynthesis, and apoptosis.

Conclusions—Deficiency of macrophage ACAT1 accelerates atherosclerosis in hypercholesterolemic apoE−/− mice but has no effect when the hypercholesterolemia is corrected by macrophage apoE expression. However, ACAT1 deletion impairs ABCA1-mediated cholesterol efflux in macrophages regardless of apoE expression. Changes in membrane stability, susceptibility to apoptosis, and inflammatory response may also be important in this process. (Circulation. 2005;111:2373-2381.)

Key Words: acyltransferases apolipoproteins apoptosis atherosclerosis cholesterol

E fficient cellular cholesterol homeostasis is critical for maintaining normal cell function. It is particularly important for arterial macrophages to protect against atherosclerosis. The homeostatic intracellular cholesterol content is regulated by cholesterol synthesis, influx, and efflux. Cholesterol is stored either as free cholesterol (FC) in the membrane or as cholesterol ester in cytoplasmic vesicles. The dynamic equilibrium between FC and cholesterol ester in the cell is tightly controlled by acyl-coenzyme A:cholesterol acyltransferase (ACAT).1–4 Two different isoforms of ACAT have been cloned in mammals.5–10 In humans, ACAT1 is expressed in most tissues, including hepatocytes, skin cells, adrenal cells, Kupffer cells, intestinal enterocytes, and macrophages; ACAT2 is expressed in the intestinal mucosa cells and hepatocytes. In mice, the tissue distribution pattern is similar to that in humans, except the major isoform in hepatocytes is ACAT2.5

ACAT1 promotes accumulation of cholesterol ester in vascular macrophages, thereby contributing to foam cell formation, a hallmark of early atherosclerosis. Because of the potential role of ACAT in atherosclerosis, numerous ACAT inhibitors have been developed and tested in animals as potential agents for the treatment of atherosclerosis, but so far, the results have been inconsistent.11,12 Nevertheless, previous work in our laboratory has shown that the deletion of ACAT1 in macrophages confers increased susceptibility to atherosclerosis in LDLR−/− mice fed a high-fat diet, raising the concern that selective disruption or complete inhibition of...
ACAT1 in macrophages can promote, rather than protect, atherosclerotic lesion formation.\textsuperscript{13} We therefore hypothesized that the negative effects of macrophage ACAT1 deficiency on plaque formation are accentuated by hypercholesterolemic conditions that lead to increased cellular cholesterol burden but may not be as important under normocholesterolemic conditions. Apolipoprotein (apo)E is a strong driver of cholesterol efflux,\textsuperscript{14} and the increased atherogenicity of apoE-deficient macrophages may be due to defective elimination of cholesterol.\textsuperscript{15,16} We and others have shown that macrophage apoE influences atherosclerosis both systemically, by controlling plasma cholesterol levels, and locally, by regulating cholesterol homeostasis in the macrophage.\textsuperscript{15,17} Thus, in the present study, we used bone marrow transplantation (BMT) to generate apoE\textsuperscript{−/−} mice with or without macrophage ACAT1 and apoE to address the effects of ACAT1 deletion on the development of atherosclerosis under both hypercholesterolemic and normocholesterol conditions, and we investigated the underlying molecular mechanisms of the accelerated atherosclerosis resulting from ACAT1 deficiency.

Methods

Animals

Six-week-old male apoE-deficient (apoE\textsuperscript{−/−}) mice were purchased from the Jackson Laboratory (Bar Harbor, Maine). ACAT-knockout (KO) and 2KO (ACAT\textsuperscript{−/−}, apoE\textsuperscript{−/−}) mice were bred in our laboratory from founders generously provided by Dr Robert V. Faresse (Gladstone Institute of Cardiovascular Disease, San Francisco, Calif). C57BL/6 mice were originally obtained from Jackson Laboratory and reproduced in our mouse colony. All mice used in this study were on C57BL/6 background and were maintained in microisolator cages on a rodent chow diet (4.5% fat; Purina Mills Inc) and autoclaved acidified water ad libitum. All experimental protocols were performed according to the guidelines of Vanderbilt University’s Institutional Animal Care and Usage Committee.

BMT Experiments

We used BMT to generate apoE\textsuperscript{−/−} mice either wild type (WT) or null for macrophage ACAT1 and apoE expression. The recipients were 6-week-old male apoE\textsuperscript{−/−} mice. We used 4 types of age-matched male donor mice: (1) WT mice (ACAT\textsuperscript{++/−}, apoE\textsuperscript{−/−}). (2) ACAT\textsuperscript{−/−} mice (ACAT\textsuperscript{−/−}, apoE\textsuperscript{−/−}), (3) ACAT\textsuperscript{−/−} mice (apoE\textsuperscript{−/−}), and (4) ACAT, apoE double-KO mice (ACAT\textsuperscript{−/−}, apoE\textsuperscript{−/−}). BMT experiments were done according to the method described previously.\textsuperscript{17} After reconstitution, the macrophages from the recipient mice are expected to have the same genotype as the donor mice and were identified accordingly: WT ACAT1, WT (ACAT\textsuperscript{++/−}, apoE\textsuperscript{−/−}), ACAT1 KO, ACAT\textsuperscript{−/−} (ACAT\textsuperscript{−/−}, apoE\textsuperscript{−/−}), apoE KO, apoE\textsuperscript{−/−} (ACAT\textsuperscript{++/−}, apoE\textsuperscript{−/−}), and 2KO (ACAT\textsuperscript{−/−}, apoE\textsuperscript{−/−}). After BMT, mice were fed a regular chow diet (4.5% fat; Purina Mills Inc) for 12 weeks.

Serum Lipid Analysis

Serum samples were collected after an overnight fast at several time points: 2 weeks before BMT and 4, 8, and 12 weeks after BMT. Serum cholesterol and triglyceride levels were determined by methods described previously.\textsuperscript{18} Serum obtained at 12 weeks after BMT was fractionated by fast-performance liquid chromatography through a Pharmacia Superose-6 column (Pharmacia Biotech Inc), and fractions were measured for total cholesterol concentration.

Quantification of Atherosclerotic Lesions in the Proximal Aorta

Twelve weeks after BMT, all recipient mice were euthanized, and the hearts with proximal aorta were harvested, embedded in OCT, and snap-frozen in liquid nitrogen for further analysis. Frozen sections (5 and 10 \(\mu\)m) were processed according to the procedures commonly used in our laboratory and described previously.\textsuperscript{17}

Lipoprotein Isolation and Modification

Human plasma was isolated from healthy volunteers. Lipoproteins were isolated by sequential ultracentrifugation with plasma density adjusted to 1.019 to 1.063 g/mL for LDL. Lipoproteins were dialyzed overnight in 0.15 mol/L NaCl and 0.3 mmol/L EDTA at pH 7.4. Acetylated LDL (acLDL) was prepared by repeatedly adding acetic anhydride (Sigma) to LDL in a sodium acetate solution and dialyzed in 0.15 mol/L NaCl and 0.3 mmol/L EDTA at 4°C for 2 days.\textsuperscript{19}

\(3^H\)-Cholesterol Efflux in Peritoneal Macrophages

Cholesterol efflux from peritoneal macrophages was determined by a modified procedure from Lin et al.\textsuperscript{20} Twelve weeks after BMT, peritoneal macrophages were collected from recipient mice 3 days after intraperitoneal injection with 3% thiglycollate. Cells were harvested by peritoneal lavage and cultured in DMEM/10% FBS at 37°C and 5% CO\textsubscript{2} overnight. The culture media was changed to DMEM/4% FBS containing 2 \(\mu\)g/mL of \(3^H\)-cholesterol (NEN) and 50 \(\mu\)g/mL acLDL, and cells were loaded for 24 hours. Monolayers were washed 3 times in serum-free DMEM/0.2% BSA and equilibrated in serum-free DMEM/0.5% BSA for 4 hours at 37°C and 5% CO\textsubscript{2}. Efflux media with serum-free DMEM/human apoA-I (10 \(\mu\)g/mL) was used to measure efflux mediated by the ABC-A1 transporter. Cells were incubated at 37°C and 5% CO\textsubscript{2}, with 100-\(\mu\)L samples removed from each well at 0, 3, 6, and 18 hours. \(3^H\)-cholesterol counts were detected with a scintillation counter. Total cellular \(3^H\)-cholesterol counts and total cellular protein mass were determined by lysing labeled cells with 0.1N NaOH. Protein concentration in the cell lysate was measured by the method of Lowry. Cholesterol efflux, calculated from the total supernatant counts, is expressed as a percentage of the total lysate counts.

Real-Time Reverse-Transcriptase Polymerase Chain Reaction for Quantification of ABCA1, ABCG1, and SR-BI gene Expression

The relative quantities of ABCA1, ABCG1, and SR-BI message were quantified by real-time reverse-transcriptase polymerase chain reaction (RT-PCR) using a previously described method developed in our laboratory.\textsuperscript{21-23} The primers and probes were synthesized by Invitrogen and ABI, respectively. TaqMan 1-step RT-PCR master mix reagent kit (ABL, P/N 4309169) was used for RT-PCR. Relative quantification of ABCA1, ABCG1, and SR-BI was normalized with 18S ribosomal RNA as an endogenous control. Data were analyzed by use of the comparative \(C_r\) method and were confirmed by standard curve method. The mRNA quantity for the calibrator (WT untreated macrophages) is expressed as 1\%^\times\%^ sample; all other quantities are expressed as the number of fold changes relative to the calibrator.

Western Blot Analysis for ABCA1, ABCG1, and SR-BI

Western blotting was performed as previously described with modifications.\textsuperscript{22} Briefly, peritoneal macrophages were isolated from WT, ACAT\textsuperscript{−/−}, apoE\textsuperscript{−/−}, and 2KO mice. Cells were cultured in either DMEM with 2% FBS (untreated) or DMEM with 2% FBS plus 50 \(\mu\)g/mL acLDL for 24 hours (treated). Cell proteins (40 \(\mu\)g) were separated by 4% to 12% Bis-Tris gel (Invitrogen). Polyclonal antibodies to ABCA1 and SR-BI were purchased from Novus Biologicals. Rabbit anti-mouse ABCG1 antibody was purchased from Alpha Diagnostic International. Mouse anti-\(\beta\)-actin antibody was obtained from Sigma.

cDNA Microarray Analysis of Peritoneal Macrophages

Total RNA was extracted from peritoneal macrophages with the Trizol reagent (Invitrogen). The isolated RNA samples were treated
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was dramatically reduced in ACAT1−/−/apoE−/−→apoE−/− and ACAT1−/−/apoE−/−→apoE−/− mice as a result of the restoration of circulating apoE and the consequent reduction in serum cholesterol (Figure 2A). The presence or absence of macrophage ACAT did not affect the extent of atherosclerosis in mice receiving apoE−/− marrow but increased lesion size in mice receiving apoE−/− marrow, suggesting that ACAT1 deficiency has no influence on atherosclerosis under normocholesterolemic conditions but has a prominent effect under hypercholesterolemic conditions (Figure 2B).

ABCA1-Mediated Cholesterol Efflux in ACAT1−/− Peritoneal Macrophages

In the presence of human apoA-I as an acceptor, cholesterol efflux was reduced ~30% to 40% in ACAT1−/− macrophages compared with ACAT1+/+ macrophages in either the presence or absence of apoE (Figure 3). Cholesterol efflux experiments were performed under mild acLDL loading conditions; under these conditions, there was no difference between the total cholesterol mass of WT and ACAT1−/− macrophages (P = 0.291; n = 3).

Real-Time Quantitative RT-PCR and Western Blot Analysis of ABCA1, ABCG1, and SR-BI

There were no significant changes in ABCG1 and SR-BI mRNA expression levels (data not shown). No significant changes were seen in ABCG1 and SR-BI protein levels as detected by Western blot and normalized to β-actin (Figure 4C and 4D). However, ABCA1 mRNA level was significantly increased by 3- to 4-fold in the 2KO and 2KO with mild acLDL loading compared with WT macrophages. ACAT−/− macrophages had a 2-fold increase in ABCA1 mRNA compared with the WT macrophages (Figure 4A). Nevertheless, ABCA1 protein levels were decreased in both ACAT1−/−-acLDL and 2KO macrophages as detected by Western blot (Figure 4B), indicating abnormal posttranscriptional regulation of ABCA1 in ACAT1−/− macrophages.

Gene Expression Analysis of ACAT1−/− Macrophages

ACAT1−/− macrophages showed a 2- to 3-fold decrease in mRNA levels of genes involved in membrane integrity (zinc finger DHHC-containing domain 5), phospholipid binding (milky fat globule–EGF factor 8 protein), functional and dynamic regulation of mitotic spindles (sperm-associated antigen 5), and a critical enzyme involved in collagen maturation (procollagen-proline, 2-oxoglutarate 4-dioxygenase) compared with WT ACAT1 macrophages. Conversely, a 2- to 3-fold increase in procollagen mRNA was observed in ACAT1−/− macrophages. The 2KO macrophages showed a gene expression pattern similar to the ACAT1−/− macrophages except for the involvement (decreased expression) of a number of genes that protect cells from oxidative damage (heme oxygenase, kynurenine 3-hydroxylase, cytochrome c oxidase) and apoptosis such as cystein proteinases (cathepsin H) (Figure 5). Gene expression profiling under acLDL loading conditions (70 µg acLDL for 16 hours) is summarized in the Table. Of note, ACAT1-deficient macrophages showed a downregulation of translation initiation factor-3 and

TUNEL Staining of Peritoneal Macrophages

Terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL) and MOMA-2 staining were performed as previously described.11 To visualize macrophages, we incubated sections with the primary antibody directed against rat MOMA-2 (Accurate Chemical & Scientific Corp) and used a TRITC-conjugated anti-rat secondary antibody (Pharmingen). For TUNEL staining, sections were incubated with deoxynucleotidyl transferase and fluorescein-labeled nucleotide mixture according to manufacturer’s specifications (In Situ Cell Death Detection Kit, Roche Diagnostics GmbH). Stained sections were analyzed with fluorescent microscopy (Olympus AX70 microscope) and an Optronics digital camera. Controls for each experiment included serial sections treated with DNase (positive control) or incubated with the labeling solution without terminal transferase (negative control).

Statistical Analysis

All results are expressed as mean±SE unless indicated otherwise. Differences between groups were assessed by use of 1-way ANOVA for comparisons among groups, followed by the Bonferroni posttest when statistical significance was detected. Atherosclerotic lesion assessment was also analyzed by 2-way ANOVA for apoE and ACAT status. Values of P < 0.05 were considered statistically significant.

Results

Serum Lipid Profile in Recipient Mice

The average serum cholesterol at baseline was between 320 and 380 mg/dL, with no significant differences between groups. As expected, the ACAT1−/−/apoE−/−→apoE−/− and ACAT1−/−/apoE−/−→apoE−/− mice had a dramatic decrease in total serum cholesterol as a result of the presence of macrophage apoE in plasma.17 However, there were no significant differences in plasma total cholesterol (Figure 1A) and triglycerides (Figure 1B) between mice receiving ACAT1−/− and those receiving ACAT1+/+ marrow. Similarly, fast-performance liquid chromatography analysis of the serum lipoproteins revealed patterns similar to previously published effects resulting from the presence or absence of macrophage apoE,17 but there was no apparent influence from the presence or absence of ACAT1 (Figure 1C).

Development of Atherosclerosis

There was a significant increase in the average lesion area (2-fold) in the proximal aorta of ACAT1−/−/apoE−/−→apoE−/− compared with ACAT1+/+/apoE−/−→apoE−/− mice. Consistent with our previous results,17 atherosclerosis
Analyses of Lesion Composition

A significant increase in apoptotic cells within the lesion area was observed in ACAT1+/apoE−/−→apoE−/− mice relative to the other recipients. The apoptotic cells appeared to be macrophages as determined by MOMA staining (Figure 6). The collagen content in the proximal aortic lesion area, as assessed by trichrome staining, was increased 3-fold in ACAT1+/apoE−/−→apoE−/− mice compared with ACAT1+/+ apoE−/−→apoE−/− mice (data not show).

Discussion

We used BMT to generate apoE−/− mice with or without macrophage ACAT1 to address the effects of ACAT1 dele-
tion on the development of atherosclerosis under both hypercholesterolemic conditions, when systemic apoE is absent, and normocholesterolemic conditions, when macrophage apoE is restored on the background of systemic apoE deficiency. We showed that ACAT1 deficiency aggravates lesion formation in the absence of macrophage apoE but has no effects when the hypercholesterolemia is corrected by macrophage apoE synthesis. To further investigate the effect of ACAT1 deficiency on macrophage cholesterol homeostasis, we performed cholesterol efflux experiments and demonstrated that ABCA1-mediated efflux to apoA-I is reduced 30% to 40% in ACAT1−/− macrophages. The reduction in cholesterol efflux was found to be independent of the presence of macrophage apoE and probably was due to the decrease in ABCA1 protein (Figure 4A). The reduction in ABCA1-mediated cholesterol efflux observed in ACAT1−/− macrophages may contribute to the increase in atherosclerosis. ABCA1 is a key player in reverse cholesterol transport and is critical in regulating cellular cholesterol homeostasis. ABCA1 is regulated both at the transcriptional level via liver X receptor and retinoid X receptor and at the posttranscriptional level via changes in trafficking and the turnover rate of ABCA1 protein. Zinc finger protein 202 (ZNF202) has been reported to suppress ABCA1 expression. Downregulation of ZNF202 could result in a loss of repression, leading to an increase in ABCA1 expression as one of the regulatory mechanisms at the transcriptional level. Our microarray data in the ACAT1-deficient macrophages show a downregulation of zinc finger DHHC containing protein 5 (Zdhhc5), a member of a recently identified zinc finger superfamily that has been implicated in transcriptional regulation of nuclear export signal and in protein-protein, protein-DNA, and protein-lipid interaction in the nucleus and cytoplasm. It is tempting to speculate that Zdhhc5 may also play a role in regulating ABCA1 expression.

The increase in ABCA1 message could also be a compensatory response to FC accumulation via liver X receptor/retinoid X receptor activation. Our results show a discordant expression between ABCA1 message and its protein, suggesting a defect in posttranscriptional regulation of ABCA1. The decrease in ABCA1 protein may be due to either an increase in protein degradation or a reduction in translation. Interestingly, reduced expression of translation initiation factor-3 (the Table). In agreement with our results, Feng and Tabas31 have shown that ABCA1-mediated cholesterol and phospholipid efflux was inhibited by ~80% in pretoxic FC-loaded macrophages and was due mainly to an increase in ABCA1 degradation. Moreover, it is possible that the downregulation of Zdhhc5 and other membrane proteins could affect ABCA1 trafficking and localization to the cell membrane, thereby leading to a reduction in ABCA1-mediated cellular cholesterol efflux. The effects of ABCA1 in macrophages resulting from ACAT1 deficiency in the presence of apoE (ACAT1−/−) are not as dramatic as in 2KO macrophages. However, even under mild loading conditions, ACAT1−/− macrophages have

Figure 2. Proximal aortic lesions 12 weeks after BMT. A, Representative 10-μmol/L cross sections of aortic root of apoE−/− recipient mice 12 weeks after BMT. Sections were stained with oil red O and hematoxylin. A, WT ACAT1 (apoE−/−, ACAT1+/−→apoE−/−); B, ACAT1 KO (apoE−/−, ACAT1−/−→apoE−/−); C, apoE KO (apoE−/−, ACAT1−/−→apoE−/−); D, 2KO (apoE−/−, ACAT1−/−→apoE−/−). B, Quantification of lesion area. Data represent mean±SEM (n=9 per group). P<0.05, **P<0.001 as determined by 1-way ANOVA to compare difference in lesion size among groups. When analyzed by 2-way ANOVA, atherosclerotic lesion as function of apoE and ACAT status, there is significant difference between apoE−/− and 2KO (P<0.01) and no significant difference between WT and ACAT−/− groups (P>0.05).

Figure 3. Macrophage cholesterol efflux to apoA-I. Twelve weeks after BMT, peritoneal macrophages from WT, ACAT1−/−, apoE−/−, and 2KO mice (n=3 for each group) were collected. Macrophages of same genotype were pooled and plated on 24-well plate. Six wells of macrophages from each genotype were loaded with 50 μg/mL acLDL and 3H-cholesterol for 24 hours and then cultured in efflux media with 10 μg/mL human apoA-I. 3H-cholesterol efflux measured as a percentage of cellular 3H-cholesterol counts showed that efflux is reduced 30% to 40% in ACAT1-KO and double-KO macrophages vs WT and apoE-KO macrophages.

Figure 4. Effect of ACAT1 deficiency on macrophage cholesterol efflux. Cells were differentiated in 24-well plate. Six wells of macrophages from each genotype were pooled and plated on 24-well plate. Six wells of macrophages from each genotype were loaded with 50 μg/mL acLDL and 3H-cholesterol for 24 hours and then cultured in efflux media with 10 μg/mL human apoA-I. 3H-cholesterol efflux measured as a percentage of cellular 3H-cholesterol counts showed that efflux is reduced 30% to 40% in ACAT1-KO and double-KO macrophages vs WT and apoE-KO macrophages.
significantly decreased ABCA1 protein, suggesting that ACAT deficiency alone could affect the ABCA1-mediated cholesterol efflux, as indicated in Figure 3. ABCG1 and SR-BI are also important mediators of cholesterol efflux and play critical roles in maintaining cellular cholesterol homeostasis. However, ACAT1 deficiency has no significant effect on either the mRNA or protein levels of both ABCG1 and SR-BI measured by real-time RT-PCR and Western blot, respectively, suggesting that the primary effect on cholesterol homeostasis resulting from ACAT1 deficiency is likely due to the downregulation of the ABCA1-mediated pathway.

The importance of macrophage apoE in the pathogenesis of atherosclerosis has been demonstrated by our previous studies and others. However, the interaction between ACAT1 and apoE in the effect of cellular cholesterol homeostasis has not been studied. Our data suggest that even though both ACAT1 and apoE have significant influence on cholesterol efflux, it is likely that such an effect is through independent mechanisms. Nevertheless, the combined deletion of apoE and ACAT1 had a greater effect on macrophage cholesterol homeostasis and resulted in a significant increase in atherogenesis compared with the deletion of apoE alone. However, the all-or-none effects of macrophage apoE on serum cholesterol make it difficult to identify possible physiological connections between these proteins. We have previously reported that the absence of either macrophage apoE or ACAT1 increases atherosclerosis in LDLR−/− mice in the presence of a more severe hypercholesterolemia than that

Figure 4. Quantification of ABCA1, ABCG1, and SR-BI in peritoneal macrophages. A, Relative quantity of ABCA1 mRNA is significantly increased in 2KO macrophages and 2KO+acLDL vs WT (P<0.001). B, C, D, Western blots of ABCA1, ABCG1, and SR-BI, respectively. Total cell lysates (40 μg) were loaded in each lane. Top, ABCA1, ABCG1, and SR-BI protein; bottom, β-actin. Relative ratios of intensity-normalized β-actin are shown on bar graph. B, ABCA1 protein in ACAT−/−-acLDL and 2KO macrophages is significantly reduced. No significant differences in ABCG1 and SR-BI protein levels are seen among groups.

Figure 5. A, cDNA microarray analysis of ACAT1−/− macrophages. Red shows fold increase in gene expression compared with ACAT1+/+ macrophages; green, fold decrease. B, Gene expression changes in double-KO macrophages vs apoE−/− macrophages.
observed in the present study.13,16 We interpret this finding to mean that the increased effect on atherogenesis resulting from absence of both macrophage ACAT1 and apoE in the apoE/H/H model is most likely not mediated by the degree of plasma cholesterol changes.

The microarray analysis of ACAT1-deficient macrophages also revealed a decreased gene expression for proteins associated with the mitotic spindle apparatus regulating mitosis (sperm-associated antigen 5), proteins required for posttranscriptional processing and metabolism of histone mRNA

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Figure 6. TUNEL staining of proximal aortic lesions. Red represents MOMA staining specific for macrophages; green, apoptotic cells. Compared with apoE−/− group, 2KO group has more positive staining for apoptosis. With higher magnification (×400), TUNEL staining is seen to localize in the macrophages.
ACAT1−/− macrophages may also be exposed to proinflammatory factors such as interleukin-1 (IL-1) receptor.68 We observed a 3-fold increase in gene expression of IL-1 receptor type I in the double-KO macrophages compared with apoE-KO macrophages (the Table). The IL-1 family comprises 4 members: IL-1α, IL-1β, IL-1 receptor antagonist (IL-1ra), and IL-18. IL-1 binds to its receptor and triggers intracellular signal transduction through a p38 mitogen-activated protein kinase–activated phosphorylation cascade. This, in turn, activates the transcription of a variety of proinflammatory genes, including the production of IL-1 itself.39 Therefore, the IL-1 family is among the most important cytokine mediators of the inflammatory responses and is thought to play a key role in the propagation of vessel wall inflammation in atherosclerosis.39 IL-1ra is an endogenous inhibitor of the IL-1 receptor, which displays affinity for IL-1R but does not induce a cellular response. IL-1ra transgenic mice have decreased atherosclerosis,40 whereas administration of FC loading–induced apoptosis in macrophages. FC loading activates the unfolded protein response in the endoplasmic reticulum, resulting in the activation of signaling pathways that promote programmed cell death.37

Our microarray data showed a 2- to 3-fold upregulation of procollagen types II and III in ACAT−/− macrophages compared with the ACAT+ macrophages. Evidently, procollagen-proline, 2-oxoglutarate-4-dioxygenase, α-subunit (P4HA1), an enzyme that plays a critical role in collagen maturation, is downregulated in ACAT+ macrophages. P4HA1 catalyzes the formation 4-hydroxyproline by hydroxylation of proline residues in the nascent procollagen protein, and the hydroxylated proline is known to be essential for the assembly of newly synthesized procollagen polypeptide chain into triple helical molecules.42 Therefore, one can speculate that the decrease in P4HA1 might result in an accumulation of procollagen peptide in the extracellular matrix of the lesion, which is supported by an increased collagen content found in the proximal aortic lesions by trichrome staining in the 2KO mice (data not show). However, it has been reported that most collagens in the lesion are made by smooth muscle cells in later stages of the lesion,43 and the relative contribution of macrophage-produced collagens in early lesion composition and remodeling needs to be further investigated.

The vascular consequences of systemic ACAT1 deficiency in hyperlipidemic mice are complex and not without controversy. We reported that both LDLR−/− and apoE−/− mice have reduced plasma lipid levels and increased lesion size when ACAT1 is absent. Then, we showed that macrophage-specific deletion of ACAT1 increases lesion size and induces apoptosis.13,44 These findings, however, are in contrast to those of a study that reported a reduction in atherosclerosis in apoE−/− mice.45 Because of the possible clinical utility of interfering with ACAT function in vivo, several inhibitors have been tested in mouse models for their effects on atherosclerosis. The results suggest that nonselective inhibition of ACAT may have a protective role in atherogenesis in animal models13 and in vitro studies in macrophages.66,67 However, selective inhibition of ACAT1 increases atherosclerosis in animal models.12 It is reasonable to speculate that perhaps the antiatherogenic effects are due to the inhibition of ACAT2, with a subsequent reduction in cholesterol absorption and plasma VLDL concentrations. This notion is also supported by the evidence that apoE−/− mice deficient in ACAT2 are resistant to atherosclerosis.48 Therefore, selective inhibition of ACAT2 has promising potential in the treatment of atherosclerosis, whereas inhibition of ACAT1 may have too profound an influence on macrophase cholesterol homeostasis to be a target of pharmacological inhibition.

Our study provides evidence of the detrimental biological effects on the vasculature after macrophase ACAT1 deletion. The underlying molecular mechanisms of disrupted cellular cholesterol homeostasis and accelerated atherosclerosis are likely due to the reduction in ABCA1-mediated cholesterol efflux and predisposition to inflammation and apoptosis.

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Yan Ru Su, Dwayne E. Dove, Amy S. Major, Alyssa H. Hasty, Branden Boone, MacRae F. Linton and Sergio Fazio

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