Available online at www.sciencedirect.com
SCIENCE DIRECT®
Biochemical and Biophysical Research Communications 331 (2005) 61–68
BBRC
www.elsevier.com/locate/ybbrc

ACAT1 deletion in murine macrophages associated with cytotoxicity and decreased expression of collagen type 3A1

Annabelle Rodriguez [a,*], M. Dominique Ashen [b], Edward S. Chen [a]

[a] Department of Medicine, Johns Hopkins University School of Medicine, Baltimore, MD 21224, USA
[b] Johns Hopkins University School of Nursing, USA

Received 15 March 2005
Available online 30 March 2005

Abstract

In contrast to some published studies of murine macrophages, we previously showed that ACAT inhibitors appeared to be anti-atherogenic in primary human macrophages in that they decreased foam cell formation without inducing cytotoxicity. Herein, we examined foam cell formation and cytotoxicity in murine ACAT1 knockout (KO) macrophages in an attempt to resolve the discrepancies. Elicited peritoneal macrophages from normal C57BL6 and ACAT1 KO mice were incubated with DMEM containing acetylated LDL (acLDL, 100 μg protein/ml) for 48 h. Cells became cholesterol enriched and there were no differences in the total cholesterol mass. Esterified cholesterol mass was lower in ACAT1 KO foam cells compared to normal macrophages ($p < 0.04$). Cytotoxicity, as measured by the cellular release of [^{14}C]adenine from macrophages, was approximately 2-fold greater in ACAT1 KO macrophages as compared to normal macrophages ($p < 0.0001$), and this was independent of cholesterol enrichment. cDNA microarray analysis showed that ACAT1 KO macrophages expressed substantially less collagen type 3A1 (26-fold), which was confirmed by RT-PCR. Total collagen content was also significantly reduced (57%) in lung homogenates isolated from ACAT1 KO mice ($p < 0.02$). Thus, ACAT1 KO macrophages show biochemical changes consistent with increased cytotoxicity and also a novel association with decreased expression of collagen type 3A1.

Keywords: Atherosclerosis; Foam cells; Cytotoxicity; ACAT

Cholesteryl ester (CE)-enriched macrophages or foam cells are a characteristic finding in atherosclerotic lesions [1]. One mechanism by which macrophages become CE-enriched is following receptor- and non-receptor mediated uptake of modified apolipoprotein B containing lipoproteins [2]. Accumulation of excess unesterified cholesterol (UC), derived from the uptake of modified lipoproteins, is converted into esterified cholesterol (EC) by the action of a key enzyme, acyl-CoA:cholesterol acyltransferase (ACAT), localized primarily in the endoplasmic reticulum [2]. Two isoforms of ACAT have been described, with type 1 being more ubiquitous in distribution (i.e., liver, kidneys, and macrophages) and type 2 found exclusively in the liver and intestine [3].

The role of ACAT in the pathogenesis of atherosclerosis is an important one. Knowledge of ACAT's critical role in foam cell formation and atherosclerosis has led to the development of numerous inhibitors of this enzyme [4]. Using an in vitro model, we previously showed that the addition of the ACAT inhibitor, 58-035, to culture medium containing acetylated LDL (acLDL) significantly lowered total cholesterol (TC) and EC accumulation, without a significant rise in UC or cellular [^{14}C]adenine release (a scientifically accepted measure of cytotoxicity) in primary human monocyte-macrophages (HMMs) [5]. We subsequently confirmed similar results with a different ACAT inhibitor, CI-1011 [6].

* Corresponding author. Fax: +1 410 550 6864.
E-mail address: arodrig5@jhmi.edu (A. Rodriguez).

0006-291X/$ - see front matter © 2005 Elsevier Inc. All rights reserved.
doi:10.1016/j.bbrc.2005.03.126

Whether using 58-035 or CI-1011 in cultured HMMs, neither inhibitor induced cytotoxicity as evidenced by the lack of significant differences in cellular [^{14}C]adenine released to the medium.

In contrast, previous studies by Warner et al. [7] suggested that in murine macrophages that accumulated UC mass following acLDL treatment and ACAT inhibition, there were significant increases in toxicity. This toxicity could be ameliorated by providing extracellular cholesterol acceptors or with the presence of the intracellular cholesterol transport inhibitor, U18666A [7].

The influence of ACAT inhibition on cell toxicity remains controversial. The ACAT1 knockout (KO) mouse provides a useful model to directly examine the effects of ACAT1 deficiency on both macrophage foam cell formation and cytotoxicity without exogenous stimulation of other pathways. The work of Meiner et al. [8] showed that ACAT1 KO macrophages exposed to acLDL had significantly less EC cholesterol accumulation but no significant differences in UC mass compared with normal macrophages. Cytotoxicity, however, was not examined in these studies.

In this study, we compare foam cell formation and cytotoxicity in peritoneal macrophages isolated from ACAT1 KO mice to those seen in normal macrophages. We show that ACAT1 deficiency does not affect total cholesterol accumulation but does lead to differences in intracellular cholesterol distribution and decreased expression of collagen type 3A1 and increased cytotoxicity.

Materials and methods

Materials. Fetal bovine serum (FBS) was purchased from Gibco. Falcon Primaria tissue culture plates were purchased from Becton–Dickinson. [1,2-^{3}H]Cholesteryl oleate (56.0 μCi/mmol) was purchased from Amersham. Specific gene primers were synthesized by Genosys. All other chemicals were of reagent grade or better.

Cell culture. Control C57BL6 adult female and male mice, which are the background strain for the ACAT1 KO mice, were purchased from Jackson Laboratories. Homozygotic ACAT1 KO mice were obtained from Dr. Robert V. Farese, Jr. (Gladstone Institute, CA). The animals were fed a normal rodent chow diet and were housed in an IACUC approved animal facility. Elicited macrophages were isolated from the peritoneal cavity by saline lavage four days after mice were initially injected with 10% thioglycollate. The mice were euthanized by placement in a CO_2 chamber, followed by cervical dislocation. Macrophages (approximately 2×10^6 cells/well) were plated on Falcon Primaria dishes (35 mm) in DMEM containing 20% fetal bovine serum (FBS). After 2 h nonadherent cells were aspirated and removed, and the monolayer was rinsed four times with serum free DMEM. The cells were then incubated overnight in DMEM containing 20% FBS. For use in experiments the medium was removed and the cells were again rinsed four times with serum free DMEM; the cells were then incubated with various experimental media. The protocol was approved by the Sinai Hospital Animal Research Committee.

Foam cell formation. Normal and ACAT1 KO macrophages were incubated with either control DMEM containing bovine serum albumin (BSA) (0.2%) or the same supplemented with acLDL (100 μg protein/ml) for 48 h. At the end of the incubation period, the medium was removed and cells were rinsed four times with cold phosphate-buffered saline. The cellular lipids were extracted with hexane:isopropranol (3:2, v/v) for 1 h at room temperature [2]. Quantitation of UC and TC mass was determined by subjecting an aliquot of the cellular lipid extract to gas liquid chromatography (GLC), using stigmasterol as an internal standard [9]. Cellular protein was measured using the Markwell modification of the Lowry procedure [10]. All values were normalized to cell protein.

Cytotoxicity assay. After cells were maintained overnight in DMEM containing 20% FBS, the medium was removed and cells were rinsed four times with serum free DMEM. Normal and ACAT1 KO macrophages were then incubated with DMEM containing BSA (0.2%) and [^{14}C]adenine (0.5 μCi/ml) for 2 h (time zero) as described by Warner et al. [7]. The medium was aspirated, cells, rinsed again with serum free medium, and then cells were incubated with either control DMEM containing BSA (0.2%) or the same supplemented with acLDL (100 μg protein/ml) for 48 h. At the end of this incubation period, the medium was removed and subjected to centrifugation at 1500 rpm at room temperature for 15 min to pellet nonadherent cells. An aliquot was taken for measurement of [^{14}C]adenine using liquid scintillation spectroscopy. The release of [^{14}C]adenine to the medium was calculated as the ([^{14}C]adenine cpm in the medium/[^{14}C]adenine in the cell at time zero) × 100. The use of [^{14}C]adenine has been shown to be a sensitive measurement of cell injury [11,12].

Cholesterol efflux during foam cell formation. Macrophages from normal and ACAT1 KO mice were exposed for 24 h to DMEM containing acLDL (100 μg protein/ml) radiolabeled with [1,2-^{3}H]cholesteryl oleate. At the end of the incubation period, the medium was collected and centrifuged at 1500 rpm for 10 min to pellet nonadherent cells. An aliquot of the medium was counted for the presence of [1,2-^{3}H]cholesterol released from the cells using liquid scintillation spectroscopy; the UC and EC fractions were separated by thin layer chromatography hexane:petroleum ether:glacial acetic acid (85:15:2, v/v). Cellular lipids were quantitated by thin layer chromatography and GLC, and all values were normalized to cell protein. This experimental design is similar to that which we used in primary human monocyte-macrophages that were exposed to ACAT inhibitors [5,6]. The rationale is to determine if cholesterol originating from acLDL is internalized, processed intracellularly, and then is eliminated by efflux to the medium and if this process is different between ACAT1 KO and normal macrophages. This experimental design differs from experiments in which HDL is the cholesterol acceptor.

cDNA microarray. Total RNA was obtained from peritoneal macrophages isolated from 10 normal and 10 ACAT1 KO mice. The macrophages were cultured for 48 h in DMEM containing 20% FBS, no additional source of cholesterol was added (such as acLDL) prior to RNA isolation. The RNA was amplified using MessageAmp RNA kit (Ambion) and labeled by direct incorporation of Cy5-dUTP or Cy3-dUTP in a reverse transcription reaction. Differentially labeled cDNAs derived from normal and ACAT1 KO mice were combined and cohybridized to microarrays containing 15,000 mouse genes. Data points with high quality scores were selected and genes demonstrating at least 2-fold changes in expression levels were identified using a dye-swap design. The microarrays were performed twice and statistical significance ($p < 0.001$) was determined using a weighted gene analysis as described by Luo et al. [13].

Reverse transcriptase-PCR assay. Total RNA was extracted from macrophages using Trizol (Sigma). Full length cDNA was generated using 1 μg of total RNA and random primers (pd(N)$_6$) as described in the First-strand cDNA synthesis kit (Amersham). RT-PCR was performed to verify reductions in collagen type 3A1: forward primer CCC AGAACATTACATACCA, and reverse primer GATTAAAACA AGATGAACAC. PCR products were run on a 6% acrylamide gel.

Collagen assay. Total collagen content was measured in lung homogenates isolated from 3 normal and 2 ACAT1 KO mice. Briefly, 1 lobe of lung was homogenized in phosphate-buffered saline using a

polytron (Brinkmann; Westbury, NY). Aliquots of the homogenates were centrifuged at 11,000*g* for 10 min and then total collagen content was measured using a colorimetric assay based on the specific binding of Sirius red F3BA dye with nondenatured collagen (Sircol Assay, Biocolor, Belfast, Northern Ireland), which is similar to a method used to quantitate collagen content in histological tissue sections [14,15]. A solution of rat tail collagen provided by the manufacturer was used as a standard for this assay. Briefly, 1 ml of Sircol dye reagent was added to 0.200 ml aliquots of lung homogenate and then incubated for 30 min at room temperature. The samples were centrifuged at 11,000*g* for 10 min and the supernatant was discarded. Dye bound to the precipitated collagen was released with a solution of 0.5 N NaOH, and the concentration of the recovered dye was determined spectrophotometrically. Total lung collagen content was normalized to total protein content.

Statistics. Unpaired Student's *t* test and Tukey–Kramer HSD (for collagen assays) were used to compare group means, with p values <0.05 considered significant.

Results

In order to compare our previous studies of foam cell formation using ACAT inhibitors in cultured HMMs [5,6], we first examined foam cell formation in normal and ACAT1 KO peritoneal macrophages by incubating cells with DMEM alone or in the same medium containing acLDL (100 μg protein/ml) for 48 h. As shown in Table 1, TC mass accumulation was similar between normal and ACAT1 KO macrophages incubated with acLDL. In addition, macrophages incubated in DMEM alone had similar TC levels. In acLDL-treated cells there was a trend towards increased UC in the ACAT1 KO macrophages but it did not reach statistical significance. In contrast, EC mass was significantly lower in ACAT1 KO macrophage foam cells ($p < 0.04$). The results were obtained from macrophages pooled from 30 normal and 34 ACAT1 KO mice.

Since we previously showed that UC efflux increased *during* foam cell formation in primary human macrophages incubated with ACAT inhibitors and acLDL, we therefore examined UC efflux in normal and ACAT1 KO macrophages after incubation with acLDL and without the presence of a known cholesterol acceptor such as HDL. Cells were incubated for 24 h with DMEM containing acLDL (100 μg protein/ml) radiolabeled with [1,2-^{3}H]cholesteryl oleate. The appearance of [1,2-^{3}H]unesterified cholesterol in the medium was used to measure cholesteryl ester hydrolysis and UC efflux. Although efflux tended to be lower in the ACAT1 KO macrophages, UC efflux was not significantly different between the two macrophages ($p = 0.21$) (Fig. 1). The results were obtained from macrophages pooled from 10 normal and 12 ACAT1 KO mice.

Because human macrophages do not appear to have increased cytotoxicity in the presence of pharmacological ACAT inhibition, we assessed cytotoxicity in normal and ACAT1 KO macrophages by first incubating cells with DMEM containing [^{14}C]adenine (0.5 μCi/ml) for 2 h (time zero) and measured adenine release after exposing cells to control medium or acLDL for an additional 48 h. As shown in Fig. 2, [^{14}C]adenine release was almost 2-fold greater in ACAT1 KO macrophages compared to normal macrophages ($p < 0.0001$). Cholesterol enrichment of cells did not significantly increase cytotoxicity over that exhibited in unloaded cells. The basal

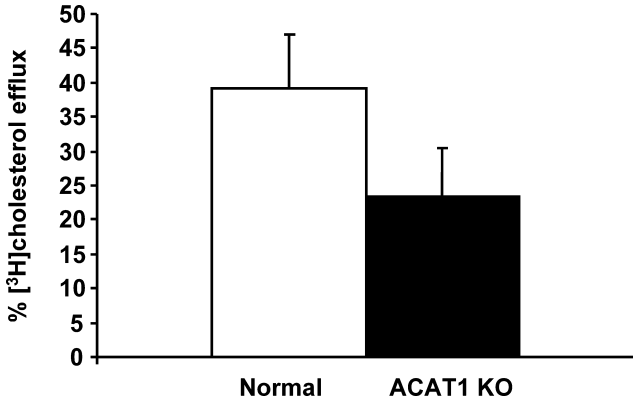

Fig. 1. Cholesterol efflux from normal and ACAT1 KO peritoneal macrophages *during* foam cell formation. Cells were exposed for 24 h to DMEM containing acLDL (100 μg protein/ml) radiolabeled with [1,2-^{3}H]cholesteryl oleate. An aliquot of the medium was counted for the presence of [1,2-^{3}H]cholesterol released from the cells using liquid scintillation spectroscopy. Cellular lipids were quantitated by thin layer chromatography, and all values were normalized to cell protein. The results were obtained from macrophages pooled from 10 normal and 12 ACAT1 KO mice.

Table 1
Intracellular cholesterol content in wild type and ACAT1 KO peritoneal macrophage foam cells

	UC (μg/mg cell protein)	EC (μg/mg cell protein)	TC (μg/mg cell protein)
Wild type, basal	16.9 ± 2.5	3.0 ± 1.5	18.0 ± 1.6
Wild type, foam cell	47.9 ± 5.0**	21.2 ± 6.3**	69.1 ± 10.8**
ACAT1 KO, basal	20.7 ± 3.6	1.9 ± 0.9	20.2 ± 3.0
ACAT1 KO, foam cell	60.3 ± 10.7**	5.0 ± 2.8*	63.8 ± 11.7**

Cellular sterol mass in normal and ACAT1 KO peritoneal macrophages. Cells were incubated with DMEM alone or in the presence of acLDL (100 μg protein/ml) for 24 h. Cellular lipids were extracted and quantitated by gas liquid chromatography. Values are means ± SE of three independent experiments (30 normal and 34 ACAT1 KO mice); FC, free or unesterified cholesterol; EC, esterified cholesterol; and TC, total cholesterol.
* $p < 0.04$ for ACAT1 KO CE compared to loaded normal macrophages.
** $p < 0.001$ compared to respective basal cells.

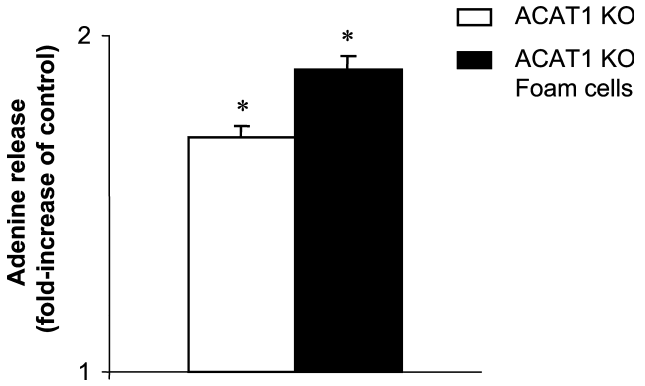

Fig. 2. Cytotoxicity in normal and ACAT1 KO macrophages as measured by the release of cellular [^{14}C]adenine to the medium. Cells were incubated with DMEM containing BSA (0.2%) and [^{14}C]adenine (0.5 μCi/ml) for 2 h (time zero). The release of [^{14}C]adenine to the medium was calculated as the ([^{14}C]adenine cpm in the medium/[^{14}C]adenine in the cell at time zero) × 100. The values are means ± SE of triplicate wells (10 normal and 4 ACAT1 KO mice). *$p < 0.0001$ compared to normal macrophages. The percent adenine release from the normal mice was 32%.

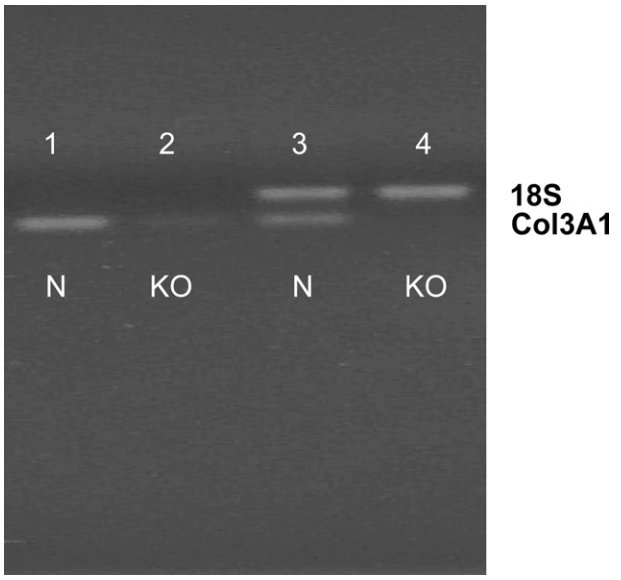

Fig. 3. Steady state RNA expression of collagen type 3A1 in ACAT1 KO and normal macrophages. Total RNA was extracted from macrophages (pooled from 10 adult ACAT1 KO and 10 adult normal mice) using Trizol (Sigma). Lane 1, col3A1 RNA in normal macrophages; lane 2, col3A1 RNA expression in ACAT1 KO macrophages; lane 3, 18S + col3A1 RNA expression in normal macrophages; and lane 4, 18S + col3A1 RNA expression in ACAT1 KO macrophages.

adenine release for normal cells was 32%, a value comparable to that reported by Kellner-Weibel et al. [16] in the murine J774 macrophage (44%).

Having found differences in cytotoxicity (that were not correlated with changes in UC mass or expression of p53 (data not shown)), we next used cDNA profiling as a screen for other differences between normal and ACAT1 KO macrophages. As shown in Table 2, the most robust decreases in cDNA expression in ACAT1 KO macrophages were for collagen type 3A1, among others many related to the extracellular matrix. Decreased steady state RNA expression was confirmed for collagen type 3A1 (Fig. 3). We also showed that total collagen quantitation was 57% lower in ACAT1 KO mice lung homogenates as compared to normal ($p < 0.02$) (Fig. 4).

Discussion

For the most part, the literature is mixed on whether ACAT1 inhibition is atherogenic or anti-atherogenic

Table 2
cDNA microarray analysis of RNA isolated from ACAT1 KO and normal mouse peritoneal macrophages

Gene	Fold-reduction in ACAT1 KO cells
M. musculus collagen α-1 COL3A1	26
Homo sapiens Jagged1	12
M. musculus 47 kDa heat shock protein	10
M. musculus secreted acidic cysteine rich glycoprotein	9
M. musculus inhibitor of DNA binding 3	8
M. musculus procollagen, type 1, α-2	7
M. musculus fibronectin	7
M. musculus biglycan	6
M. musculus exportin 4	5
	Fold-increased in ACAT1 KO cells
M. musculus for immunoglobulin mu	7
M. musculus asparagines synthetase	6
M. musculus methylenetetrahydrofolate dehydrogenase	3
M. musculus ribosomal protein S6 kinase	3
M. musculus HAS-A gene for heat stable antigen	3
M. musculus syndecan	3

Partial list, demonstrating the most robust responses.
$p < 0.001$ compared to normal macrophages.

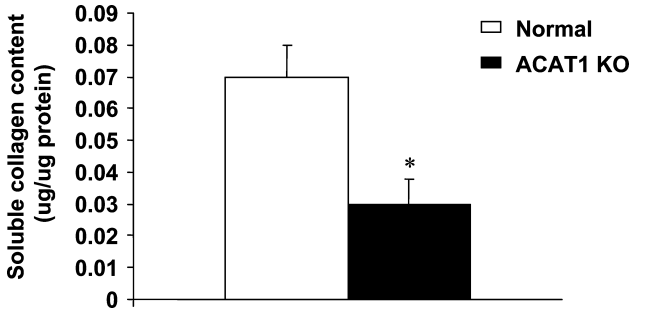

Fig. 4. Total collagen content in lung homogenates isolated from ACAT1 KO and normal mice. Collagen content was measured in lung homogenates isolated from 3 normal mice and 2 ACAT1 KO mice. *$p < 0.02$ compared to normal mice.

[17–22]. A concern has been raised regarding ACAT inhibition in atherosclerotic disease due, in large part, to two pieces of evidence: the correlation of cytotoxicity (based on increased adenine release) and excess UC mass accumulation in murine macrophage foam cells exposed to these compounds [23,24] and the findings from the double knockout animals showing increased cutaneous xanthomas in two studies [25,26] and increased atherosclerosis in one study [27]. Our studies with HMMs showed that ACAT inhibitors might be anti-atherogenic, without evidence for increased adenine release [5,6]. Moreover, human subjects exposed to the ACAT inhibitor, CI-1011, appeared to have tolerated the medication without serious adverse events and did show significant reductions in serum triglyceride levels [28].

Part of the differences in conclusions from the various studies could be the result of differences between mouse and human macrophages. For this reason, our current study compared and contrasted our previously reported studies of the apparent benefit of ACAT inhibition on foam cell formation, cholesterol efflux, and reduced cytotoxicity in cultured primary human macrophages with the same measures in ACAT1 KO macrophages. Our studies suggest that ACAT1 KO peritoneal macrophages may become enriched in unesterified cholesterol. While UC levels never reached statistically higher levels than in wild type macrophages, a definite trend was seen and the ratio of UC to EC changed dramatically. The results are consistent with those reported by Meiner et al. [8]. The results of UC efflux during foam cell formation did not show significant differences between normal and ACAT1 KO peritoneal macrophage foam cells.

It has been suggested that cytotoxicity increases in the presence of ACAT inhibition due to accumulation of UC mass, particularly in the plasma membrane compartment [29]. However, recent results by Feng et al. [30] suggested that cholesterol enrichment of the endoplasmic reticulum (ER), and not that of the plasma membrane, increased apoptosis in murine macrophages. Nonetheless, we did observe that cytotoxicity was significantly increased in ACAT1 KO macrophages compared to normal cells under conditions in which neither cells were cholesterol enriched. This indicated that ACAT1 deficiency is associated with cytotoxicity but this occurred independently of cholesterol enrichment. Kellner-Weibel et al. [29] found that cytotoxicity induced by ACAT inhibition in primary mouse peritoneal macrophage foam cells could be diminished in the presence of a modulator of intracellular cholesterol transport inhibitor, U18666A, also suggesting that the location of excess intracellular UC mass influences cytotoxicity. It should also be noted that the histology of atherosclerotic lesions in ACAT1 KO mice, with either an apolipoprotein $E^{-/-}$ or LDL receptor$^{-/-}$ background, showed an altered composition [25]. Investigators showed a paucity of macrophages in aortic lesions from the double knockout animals, including decreased cholesterol crystals [25]. It might be possible that the cytotoxicity observed in ACAT1 KO macrophages leads to increased cell death and this accounted for the dearth of cells noted in advanced atherosclerotic lesions; this was suggested by Fazio et al. [27], in which LDL receptor$^{-/-}$ mice transplanted with ACAT1$^{-/-}$ macrophages showed increased TUNEL staining in arterial lesions.

Although we found increased cytotoxicity, the levels were not correlated to cholesterol enrichment of cells. This lack of correlation of cytotoxicity by ACAT inhibition with cholesterol enrichment contrasts with the studies by Yao and Tabas [31], in which investigators examined the effects of free cholesterol loading on apoptosis in peritoneal macrophages isolated from C57BL6 mice. The authors exposed cells for 9 h to control medium, acLDL, the ACAT inhibitor 58-035, and acLDL plus 58-035. Apoptosis (as determined by annexin V staining) was elevated by 12% in cells incubated with acLDL plus 58-035 but was negligible (less than 1%) in cells incubated with control medium, acLDL, or 58-035 alone. Clearly, the effects of ACAT inhibition on cell cytotoxicity, in the presence or absence of cholesterol enrichment, appear to depend on whether ACAT is inhibited pharmacologically or by a genetic mutation, and perhaps the duration of exposure to the ACAT inhibitor alone. In fact, Warner et al. [7] had previously shown that adenine release from murine macrophages increased with duration of exposure to ACAT inhibitors, with values approximately 2-fold greater in cells exposed to ACAT inhibition for longer than 36 h; a value similar to the 2-fold increase noted in the ACAT1 KO cells.

Therefore, in order to better define the possible causes or effects of ACAT deficiency on cytotoxicity, we used cDNA microarrays to investigate effects of ACAT1 deficiency on gene expression in macrophages that had not been cholesterol enriched. As shown in Table 2, many of the genes that were substantially reduced or increased in ACAT1 KO macrophages are associated with the extracellular matrix (i.e., collagen, procollagen,

fibronectin, and syndecan), while others such as exportin 4 are involved in nucleocytoplasmic transportation of RNA [32]. Expression of Jagged1 was also substantially reduced in ACAT1 KO macrophages but what direct effect ACAT1 exerts on Jagged1 function remains unknown. Jagged1 is a cell surface ligand that binds to Notch receptors (important in intercellular signaling and vasculogenesis [33]) and its expression in macrophages is upregulated by macrophage colony-stimulating factor, granulocyte-macrophage colony-stimulating factor, and interleukin-3 [34]. Additional studies have suggested an integral role of Jagged1 in cell–cell and cell–matrix interactions [35,36]. Further work is needed to elucidate the relationship between ACAT1, collagen type 3A1, and cytotoxicity.

A possible link between collagen and measures of cellular inflammation was shown in a study by Ding et al. [37] in which these investigators found that sera collected from Sprague–Dawley rats fed oral collagen type II reduced production of interleukin-1, tumor necrosis factor, nitric oxide, and malondialdehyde in synoviocytes. In addition, a link between ACAT and ECM regulation might be possible given that another hepatic receptor, low-density lipoprotein-related protein, has been shown to be regulated by ECM [38]. We know that macrophages from ACAT1 KO mice had reduced RNA expression of Jagged1, hsp 47, procollagen I, fibronectin, biglycan, and exportin 4, among other gene transcripts. It might be possible that reduced col3A1 (and perhaps procollagen I) expression is related to alterations in hsp47 expression, a protein present in the endoplasmic reticulum that plays a vital role in collagen processing [39]. Other considerations include the fact that ACAT deficiency might decrease the activity, but not the expression, of enzymes known to regulate the expression of col3A1. For instance, Rosenbloom et al. [40] have shown that inhibition of geranylgeranyltransferase I resulted in inhibition of collagen type I and type III expression. Others have shown that activation of enzymes involved in TGF-β signaling pathways (phosphatidylcholine-specific phospholipase C, protein kinase C-δ, and p38) was important in the transcription of fibronectin [41]. Kuchich et al. [42] found that geranylgeranyltransferase I and acyl transferase were required in the TGF-β stimulated pathway for elastin. These investigators found that cerulenin, an inhibitor of protein acylation, significantly reduced elastin RNA levels in fetal human lung fibroblasts following TGF-β stimulation [42]. Thus, it might also be plausible that ACAT deficiency might alter the activity of key enzymes involved in TGF-β signaling pathways and their effect on ECM protein expression.

Lastly, a recent report by Dove et al. [43] has shown that ACAT1 deficiency is associated with decreased cholesterol efflux and altered cellular morphology. The experimental designs between our study and Dove et al. are significant in a number of respects: (1) we examined cholesterol efflux *during* foam cell formation (similar to studies conducted with ACAT inhibitors and human macrophages) and not during apoA-1 mediated cholesterol efflux and (2) we did not use 1% fetal bovine serum (which would contain HDL) during the adenine cytotoxicity assays. While cholesterol efflux during foam cell formation was not significantly different between ACAT1 KO and normal cells, the trend was lower in ACAT1 KO cells, suggesting an overall agreement between Dove et al., and our studies. We showed increased cytotoxicity in the ACAT1 KO cells in contrast to Dove et al., and believe this difference resides in the presence of FBS in their experimental design.

In summary, our studies have shown differences in foam cell formation, cholesterol efflux, and cytotoxicity between HMMs exposed to ACAT inhibitors and ACAT1 KO macrophages. Studies of ACAT inhibition in cultured murine macrophage foam cells have shown evidence of cytotoxicity, as have our present studies with peritoneal macrophages isolated from ACAT1 KO mice but without the need to induce cholesterol enrichment. In addition, collagen expression was reduced in macrophages and lung homogenates from the ACAT1 KO mouse. Thus, ACAT1 KO macrophages show biochemical changes consistent with increased cytotoxicity and also a novel association with decreased expression of collagen type 3A1.

Acknowledgments

This work was supported by an Atorvastatin Research Award. We thank Jun Luo, MD, for assistance with the cDNA microarrays.

References

[1] R. Ross, The pathogenesis of atherosclerosis: an update, N. Engl. J. Med. 314 (1986) 488–500.
[2] M.S. Brown, Y.K. Ho, J.L. Goldstein, The cholesteryl ester cycle in macrophage foam cells, J. Biol. Chem. 255 (1980) 9344–9352.
[3] K.F. Buhmamn, M. Accad, R.V. Farese Jr., Mammalian acyl-CoA:cholesterol acyltransferases, Biochim. Biophys. Acta 1529 (2000) 142–154.
[4] H.T. Lee, D.R. Sliskovic, J.A. Picard, B.D. Roth, W. Wieranga, J.L. Hicks, R.F. Bousley, K.L. Hamelehle, R. Homan, C. Speyer, R.L. Stanfield, B. Krause, Inhibitors of acyl-CoA:cholesterol *O*-acyl transferase (ACAT) as hypocholesterolemic agents, J. Med. Chem. 39 (1996) 5031–5034.
[5] A. Rodriguez, P.S. Bachorik, S.-B. Wee, Novel effects of the acyl-coenzyme A:cholesterol acyltransferase inhibitor 58-035 on foam cell development in primary human monocyte-derived macrophages, Arterioscler. Thromb. Vasc. Biol. 19 (1999) 2199–2206.
[6] A. Rodriguez, D.C. Usher, Anti-atherogenic effects of the acyl-CoA:cholesterol acyltransferase inhibitor, avasimibe (CI-1011), in cultured primary human macrophages, Atherosclerosis 161 (2002) 45–54.

[7] G.J. Warner, G. Stoudt, M. Bamberger, W.J. Johnson, G.H. Rothblat, Cell toxicity induced by inhibition of acyl coenzyme A:cholesterol acyltransferase and accumulation of unesterified cholesterol, J. Biol. Chem. 270 (1995) 5772–5778.
[8] V.L. Meiner, S. Cases, H.M. Myers, E.R. Sande, S. Bellosta, M. Schambelan, R.E. Pitas, J. McGuire, J. Herz, R.V. Farese Jr., Disruption of the acyl-CoA:cholesterol acyltransferase gene in mice: evidence suggesting multiple cholesterol esterification enzymes in mammals, Proc. Natl. Acad. Sci. USA 93 (1996) 14041–14046.
[9] T.T. Ishikawa, J. MacGee, J.A. Morrison, C.J. Glueck, Quantitative analysis of cholesterol in 5 to 20 microliters of plasma, J. Lipid Res. 15 (1974) 286–291.
[10] M.K. Markwell, S.M. Haas, L.L. Bieber, N.E. Tolbert, A modification of the Lowry procedure to simplify protein determination in membrane and lipoprotein samples, Anal. Biochem. 87 (1978) 206–210.
[11] V.C. Reid, C.E. Brabbs, M.J. Mitchinson, Cellular damage in mouse peritoneal macrophages exposed to cholesteryl linoleate, Atherosclerosis 92 (1992) 251–260.
[12] V. Shirhatti, G. Krishna, A simple and sensitive method for monitoring drug-induced cell injury in cultured cells, Anal. Biochem. 147 (1985) 410–418.
[13] J. Luo, D.J. Duggan, Y. Chen, J. Sauvageot, C.M. Ewing, M.L. Bittner, J.M. Trent, W.B. Isaacs, Human prostate cancer and benign prostatic hyperplasia: molecular dissection by gene expression profiling, Cancer Res. 61 (2001) 4683–4688.
[14] A. Lopez-DeLeon, M. Rojkind, A simple micromethod for collagen and total protein determination in formalin-fixed paraffin-embedded sections, J. Histochem. Cytochem. 33 (1985) 737–743.
[15] M. Huang, S. Sharma, L.X. Zhu, M.P. Keane, J. Luo, L. Zhang, M.D. Burdick, Y.Q. Lin, M. Dohadwala, B. Gardner, R.K. Batra, R.M. Strieter, S.M. Dubinett, IL-7 inhibits fibroblast TGF-beta production and signaling in pulmonary fibrosis, J. Clin. Invest. 109 (2002) 931–937.
[16] G. Kellner-Weibel, S.J. Luke, G.H. Rothblat, Cytotoxic cellular cholesterol is selectively removed by apoA-I via ABCA1, Atherosclerosis 171 (2003) 235–243.
[17] D.J.M. Delsing, E.H. Offerman, W. van Duyvenvoorde, H. van Der Boom, E.C. de Wit, M.J. Gijbels, A. van Der Laarse, J.W. Jukema, L.M. Havekes, H.M. Princen, Acyl-CoA:cholesterol acyltransferase inhibitor avasimibe reduces atherosclerosis in addition to its cholesterol-lowering effect in apoE3-Leiden mice, Circulation 103 (2001) 1778–1786.
[18] J. Kusunoki, D.K. Hansoty, K. Aragane, J.T. Fallon, J.J. Badimon, E.A. Fisher, Acyl-CoA:cholesterol acyltransferase inhibition reduces atherosclerosis in apolipoprotein E-deficient mice, Circulation 103 (2001) 2604–2609.
[19] T.M.A. Bocan, S.B. Mueller, E.Q. Brown, P. Lee, M.J. Bocan, T. Rea, M.E. Pape, HMG-CoA reductase and ACAT inhibitors act synergistically to lower plasma cholesterol and limit atherosclerotic lesion development in the cholesterol-fed rabbit, Atherosclerosis 139 (1998) 21–30.
[20] R.J. Nicolosi, T.A. Wilson, B.R. Krause, The ACAT inhibitor, CI-1011 is effective in the prevention and regression of aortic fatty streak area in hamsters, Atherosclerosis 137 (1998) 77–85.
[21] T. Chiwata, K. Aragane, K. Fujinami, K. Kojima, S. Ishibashi, N. Yamada, J. Kusunoki, Direct effect of an acyl-CoA:cholesterol acyltransferase inhibitor, F-1394, on atherosclerosis in apolipoprotein E and low density lipoprotein receptor double knockout mice, Br. J. Pharmacol. 133 (2001) 1005–1012.
[22] S. Perrey, C. Legendre, A. Matsuura, C. Guffroy, J. Binet, S. Ohbayashi, T. Tanaka, J.C. Ortuno, T. Matsukura, T. Laugel, P. Padovani, F. Bellamy, A.D. Edgar, Preferential pharmacological inhibition of macrophage ACAT increases plaque formation in mouse and rabbit models of atherogenesis, Atherosclerosis 155 (2001) 359–370.
[23] G. Kellner-Weibel, Y.J. Geng, G.H. Rothblat, Cytotoxic cholesterol is generated by the hydrolysis of cytoplasmic cholesteryl ester and transported to the plasma membrane, Atherosclerosis 146 (1999) 309–319.
[24] G. Kellner-Weibel, W.G. Jerome, D.M. Small, G.J. Warner, J.K. Stoltenborg, M.A. Kearney, M.H. Corjay, M.C. Phillips, G.H. Rothblat, Effects of intracellular free cholesterol accumulation on macrophage viability, Arterioscler. Thromb. Vasc. Biol. 18 (1998) 423–431.
[25] M. Accad, S.J. Smith, D.L. Newland, D.A. Sanan, L.E. King, M.F. Linton, S. Fazio, R.V. Farese, Massive xanthomatosis and altered composition of atherosclerotic lesions in hyperlipidemic mice lacking acyl CoA:cholesterol acyltransferase 1, J. Clin. Invest. 105 (2000) 711–719.
[26] H. Yagyu, T. Kitamine, J. Osuga, R. Tozawa, Z. Chen, Y. Kaji, T. Oka, S. Perrey, Y. Tamura, K. Ohashi, H. Okazaki, N. Yahagi, F. Shionoiri, Y. Iizuka, K. Harada, H. Shimano, H. Yamashita, T. Gotoda, N. Yamada, S. Ishibashi, Absence of ACAT-1 attenuates atherosclerosis but causes dry eye and cutaneous xanthomatosis in mice with congenital hyperlipidemia, J. Biol. Chem. 275 (2000) 21324–21330.
[27] S. Fazio, A.S. Major, L.L. Swift, L.A. Gleaves, M. Accad, M.F. Linton, R.V. Farese Jr., Increased atherosclerosis in LDL receptor-null mice lacking ACAT1 in macrophages, J. Clin. Invest. 107 (2001) 163–171.
[28] W. Insull, M. Koren, J. Davignon, D. Sprecher, H. Schrott, L.M. Keilson, A.S. Brown, C.A. Dujovne, M.H. Davidson, R. McLain, T. Heinonen, Efficacy and short-term safety of a new ACAT inhibitor, avasimibe, on lipids, lipoproteins, and apolipoproteins in patients with combined hyperlipidemia, Atherosclerosis 157 (2001) 137–144.
[29] G. Kellner-Weibel, P.G. Yancey, W.G. Jerome, T. Walser, R.P. Mason, M.C. Phillips, G.H. Rothblat, Crystallization of free cholesterol in model macrophage foam cells, Arterioscler. Thromb. Vasc. Biol. 19 (1999) 1891–1898.
[30] B. Feng, P.M. Yao, Y. Li, C.m. Devlin, D. Zhang, H.P. Harding, M. Sweeney, J.X. Rong, G. Kuriakose, E.A. Fisher, A.R. Marks, D. Ron, I. Tabas, The endoplasmic reticulum is the site of cholesterol-induced cytotoxicity in macrophages, Nat. Cell Biol. 5 (2003) 781–792.
[31] P.M. Yao, I. Tabas, Free cholesterol loading of macrophages induces apoptosis involving the Fas pathway, J. Biol. Chem. 275 (2000) 23807–23813.
[32] G. Lipowsky, F.R. Bishcoff, P. Schwarzmaier, R. Kraft, S. Kostka, E. Hartmann, U. Kutay, D. Gorlich, Exportin 4: a mediator of a novel nuclear export pathway in higher eukaryotes, EMBO J. 19 (2000) 4362–4371.
[33] A.H. Campos, W. Wang, M.J. Pollman, G.H. Gibbons, Determinants of Notch-3 receptor expression and signaling in vascular smooth muscle cells: implications in cell-cycle regulation, Circ. Res. 91 (2002) 999–1006.
[34] K. Nomaguchi, S. Suzu, M. Yamada, H. Hayasawa, K. Motoyoshi, Expression of Jagged1 gene in macrophages and its regulation by hematopoietic growth factors, Exp. Hematol. 29 (2001) 850–855.
[35] V. Lindner, C. Booth, I. Prudovsky, D. Small, T. Maciag, L. Liaw, Members of the Jagged/Notch gene families are expressed in injured arteries and regulate cell phenotype via alterations in cell–matrix and cell–cell interactions, Am. J. Pathol. 159 (2001) 875–883.
[36] S.E. Bell, A. Mavila, R. Salazar, K.J. Bayless, S. Kanagala, S.A. Maxwell, G.E. Davis, Differential gene expression during capillary morphogenesis in 3D collagen matrices: regulated expression of genes involved in basement membrane matrix assembly, cell cycle

progression, cellular differentiation and G-protein signaling, J. Cell Sci. 114 (2001) 2755–2773.
[37] C.H. Ding, Q. Li, Z.Y. Xiong, A.W. Zhou, G. Jones, S.Y. Xu, Oral administration of type II collagen suppresses pro-inflammatory mediator production of synoviocytes in rats with adjuvant arthritis, Clin Exp Immunol 132 (2003) 416–423.
[38] S. Schmoelzl, S.J. Benn, J.E. Laithwaite, S.J. Greenwood, W.S. Marshall, N.A. Munday, D.J. FitzGerald, J. LaMarre, Expression of hepatocyte low-density lipoprotein receptor-related protein is post-transcriptionally regulated by extracellular matrix, Lab. Invest. 78 (1998) 1405–1413.
[39] E.F. Rocnik, E. van der Veer, H. Cao, R.A. Hegele, J.G. Pickering, Functional linkage between the endoplasmic reticulum protein Hsp47 and procollagen expression in human vascular smooth muscle cells, J. Biol. Chem. 277 (2002) 38571–38578.
[40] J. Rosenbloom, B. Saitta, S. Gaidarova, N. Sandorfi, J.C. Rosenbloom, W.R. Abrams, A.D. Hamilton, S.M. Sebti, U. Kucich, S.A. Jimenez, Inhibition of type I collagen gene expression in normal and systemic sclerosis fibroblasts by a specific inhibitor of geranylgeranyltransferase I, Arthritis Rheum. 43 (2000) 1624–1632.
[41] U. Kucich, J.C. Rosenbloom, G. Shen, W.R. Abrams, A.D. Hamilton, S.M. Sebti, J. Rosenbloom, TGF-β1 stimulation of fibronectin transcription in cultured human lung fibroblasts requires active geranylgeranyltransferase I, phosphatidylcholine-specific phospholipase C, protein kinase C-δ, and p38, but not erk1/erk2, Arch. Biochem. Biophys. 374 (2000) 313–324.
[42] U. Kuchich, J.C. Rosenbloom, G. Shen, W.R. Abrams, M.A. Blaskovich, A.D. Hamilton, J. Ohkanda, S.M. Sebti, J. Rosenblood, Requirement for geranylgeranyltranferase I and acyl transferase in the TGF-β-stimulated pathway leading to elastin mRNA stabilization, Biochem. Biophys. Res. Commun. 252 (1998) 111–116.
[43] D.E. Dove, Y.R. Su, W. Zhang, W.G. Jerome, L.L. Swift, M.R. Linton, S. Fazio, ACAT1 deficiency disrupts cholesterol efflux and alters cellular morphology in macrophages, Arterioscler. Thromb. Vasc. Biol. 25 (2005) 128–134.