ACAT Inhibition Reduces the Progression of Preexisting, Advanced Atherosclerotic Mouse Lesions Without Plaque or Systemic Toxicity


Objective—Acyl-CoA:cholesterol acyltransferase (ACAT) converts cholesterol to cholesteryl esters in plaque foam cells. Complete deficiency of macrophage ACAT has been shown to increase atherosclerosis in hypercholesterolemic mice because of cytotoxicity from free cholesterol accumulation, whereas we previously showed that partial ACAT inhibition by Fujirebio compound F1394 decreased early atherosclerosis development. In this report, we tested F1394 effects on preestablished, advanced lesions of apolipoprotein-E–deficient mice.

Methods and Results—Apolipoprotein-E–deficient mice on Western diet for 14 weeks developed advanced plaques, and were either euthanized (Baseline), or continued on Western diet with or without F1394 and euthanized after 14 more weeks. F1394 was not associated with systemic toxicity. Compared with the baseline group, lesion size progressed in both groups; however, F1394 significantly retarded plaque progression and reduced plaque macrophage, free and esterified cholesterol, and tissue factor contents compared with the untreated group. Apoptosis of plaque cells was not increased, consistent with the decrease in lesional free cholesterol, no increase in plaque necrosis, and unimpaired efferocytosis (phagocytic clearance of apoptotic cells). The effects of F1394 were independent of changes in plasma cholesterol levels.

Conclusion—Partial ACAT inhibition by F1394 lowered plaque cholesterol content and had other antiatherogenic effects in advanced lesions in apolipoprotein-E–deficient mice without overt systemic or plaque toxicity, suggesting the continued potential of ACAT inhibition for the clinical treatment of atherosclerosis, in spite of recent trial data. (Arterioscler Thromb Vasc Biol. 2013;33:00-00.)

Key Words: atherosclerosis ■ foam cells ■ genetically altered mice ■ lipoproteins ■ pharmacology

Acyl-CoA:cholesterol acyltransferase (ACAT) converts cholesterol to cholesteryl esters and plays important roles in lipoprotein assembly, dietary cholesterol absorption, and intracellular cholesterol metabolism.1 ACAT exists in 2 forms, ACAT1 and ACAT2. ACAT2 is expressed in the liver, and indirectly contributes to coronary artery disease by influencing the content of cholesteryl ester (CE) on the atherogenic lipoprotein particles VLDL and LDL.2-4 Another important role for ACAT in cardiovascular disease is that in macrophages and smooth muscle cells in the arterial wall, CEs produced by ACAT1 accumulate leading to formation of foam cells, whose presence is a hallmark of atherosclerotic lesions and whose deranged metabolism exacerbate the inflammatory milieu within a plaque.5,6

Complete deficiency of macrophage ACAT results in increased atherosclerotic lesions in hypercholesterolemic mouse models (LDL receptor or apolipoprotein-E–deficient [apoE−/−] mice) because of the cytotoxicity from free cholesterol (FC) accumulation in cells and tissues.6 Early studies attributed this toxicity to either damage to the plasma membrane (because excess membrane FC, which ordinarily can be safely stored in the more inert cytosolic CE lipid droplets, was now retained), or from the formation of FC intracellular crystals.7,8 More recent studies, first by Tabas and colleagues and then others,9-11 have shown that a component of the cellular cytotoxicity is ER-stress from the accumulation of FC in the ER membrane, ultimately resulting in apoptosis in vitro and in vivo. In recent reviews, it has been proposed that in advanced plaques, there is diminution of ACAT activity and apoptotic cell clearance, so that in the face of persistent hyperlipidemia, foam cells become progressively overloaded.
with FC, undergo ER-stress and apoptosis, and then add to the necrotic core.15–17 Implicit in this scenario is that the rate of efflux of FC is insufficient to maintain subtoxic cellular levels.

In previous studies, we showed that for predominately foam cell-rich lesions, partial ACAT inhibition by Fujirebio compound F1394 was nontoxic and decreased atherosclerosis progression in apoE\(^{-/-}\) mice, when they were treated before lesion initiation.5 To simulate the scenario summarized above for more advanced plaques, in the present study, we have allowed plaques to develop to an advanced stage (so that they contain cholesterol clefts and lipid cores). Then, the mice were fed either Western diet (WD) or WD+F1394 to partially inhibit ACAT for the next 14 weeks. As will be described, F1394 treatment led to a delay in further progression of atherosclerotic lesions, as well as to other benefits, including a decrease in lesional FC, without evidence of increased foam cell apoptosis, impaired effecrocytosis, greater plaque necrosis, or signs of systemic toxicity.

**Materials and Methods**

**Experimental Design and Animals**

All experimental procedures in animals were performed with protocols approved by either the Mount Sinai School of Medicine or the NYU School of Medicine Animal Care and Use Committee. Compound F1394 was supplied by Fujirebio Inc. (Tokyo, Japan). Male apoE\(^{-/-}\) mice (n=47) were weaned at the age of 4 weeks onto a 21% (wt/wt) fat, 0.15% cholesterol Western-type diet (WD; catalogue No. 100244, Dyets Inc) and fed with this diet for 14 weeks to develop advanced lesions containing necrotic lipid cores and cholesterol clefts in the aortic roots (AHA class IV).15 These mice were then divided into 3 groups: 1 group was euthanized to obtain lesion status before F1394 diet began (n=16, Baseline); and the other 2 groups were continued on WD with (n=15, Treatment, 900 mg/kg diet; dose based on our previous study) or without (n=16, No Treatment; ie, control) supplementation of F1394 for additional 14 weeks and euthanized.

**Measurement of Plasma Lipids and Lipoproteins**

Plasma samples were collected at euthanization for all groups. The HDL fraction was isolated by ultracentrifugation, as previously described.19 Total cholesterol (TC) and HDL-cholesterol (HDL-C) were then analyzed using commercial kits (Sigma cholesterol reagent kit).

Two plasma samples (pooled from 2 mice) from the F1394 and No Treatment group were separated via fast protein liquid chromatography. The lipoproteins eluted approximately at fractions 29 to 38 as previously described. Microsomal protein, 1 mg of BSA, and 50 nmol of FC in 45% (wt/vol) \(\beta\)-cyclodextrin were mixed together, and subsequently incubated for 30 minutes at 37°C. Then, 30 nmol of \([^{14}C]\) oleoyl-CoA was added to the mix and incubated for 10 minutes. The reaction was stopped by the addition of 2:1 chloroform–methanol. After the phases separated, the organic phase was removed and applied to a thin layer chromatography plate. The CE band was scraped, suspended in scintillation fluid, and counted for \(^{14}C\) radioactivity.

**Aortic Lipid Analysis**

The TC, FC, and CE content of aortas (n=6 or 7) were measured according to methods described by Willner et al.23 Briefly, all adherent adipose and connective tissue was removed from the formalin-prepared aortas. The lipids were extracted in 2:1 chloroform:methanol, with 5\(\mu\)l \(\beta\)-cyclodextrin added as an internal standard. Gas chromatography yielded the concentration of FC. After saponification, TC was determined by gas chromatography. Aortic CE was subsequently determined by subtracting FC from TC, and multiplying the difference by 1.67.24 Protein was determined by the method of Lowry on the delipidated aortas following digestion in 1N sodium hydroxide.

**ACAT Assays**

Flash-frozen liver samples from the Treatment group (n=3) and the No Treatment group (n=3) were used to determine ACAT activity. Microsomes were isolated from the liver samples, as previously described by Carr et al.25 Microsomal protein concentration was measured by the method of Lowry et al.26 A 50-\(\mu\)l aliquot of microsomal protein was used to determine ACAT activity, as previously described.21 Briefly, microsomal protein, 1 mg of BSA, and 50 nmol of FC in 45% (wt/vol) \(\beta\)-cyclodextrin were mixed together, and subsequently incubated for 30 minutes at 37°C. Then, 30 nmol of \([^{14}C]\) oleoyl-CoA was added to the mix and incubated for 10 minutes. The reaction was stopped by the addition of 2:1 chloroform–methanol. After the phases separated, the organic phase was removed and applied to a thin layer chromatography plate. The CE band was scraped, suspended in scintillation fluid, and counted for \(^{14}C\) radioactivity.

For harvesting tissue specimens, mice were exsanguinated and perfusion-fixed in 4% paraformaldehyde (at 100 mm Hg, 5 min). The heart was transected at the lower poles of the atria and processed for paraffin embedding. Serial 5-\(\mu\)m sections (4 per mouse for all section-based assays) were taken from paraffin-embedded aortic roots and stained with combined Masson trichome-elastic stain.19,28 For immunohistochemical analyses, 5-\(\mu\)m-thick formalin-fixed, paraffin-embedded sections were deparaffinized and hydrated with PBS, then incubated with 3% hydrogen peroxide, washed with PBS, and subsequently exposed to blocking serum (normal horse serum, normal rabbit serum, normal goat serum; Vector Laboratories, Burlingame, CA). The primary antibody (rat antimouse CD68 ABD Serotec; rabbit monoclonal CCR7 (Y59); rat antimouse VCAM-1/CD106 Southern Biotechnology) was applied overnight at 4°C, as previously described.29 Immunodetection was performed using biotinylated horse antimouse secondary (Vector Laboratories, Burlingame, CA), peroxidase-labeled streptavidin (Jackson Immuno Research, West Grove, PA), and visualized using the Nova Red substrate (Vector Laboratories, Burlingame, CA). The slides were then counterstained in hematoxylin. The appropriate positive control was used, and the antibody was omitted for the negative control.

For Tdt-mediated dUTP nick-end labeling (TUNEL) staining to detect apoptotic cells, the hydrated sections were incubated with 3% hydrogen peroxide and subsequently treated following the protocol of an apoptosis detection kit (ApopTag® Peroxidase in situ apoptosis detection kit; Millipore, Billerica, MA). Staining was visualized using DAB (DAKO) and slides were counterstained with methyl green.

Apoptotic cells in atherosclerotic lesions were detected by TUNEL, after proteinase K treatment. Stringency methods of Kockx were followed to avoid nonspecific staining.22 Nuclei were counterstained, and the sections were imaged and quantified by microscopy and Image J software in a blinded manner. For percentage of TUNEL-positive cells, TUNEL+ nuclei were divided by total intimal nuclei that were counted from serial sections along the aortic root. Percentage of necrosis was quantified as hematoxylin- and eosin-negative intimal area, as previously described.19,28

Microscopic images from stained sections were captured by a Sony 3CCD Video camera attached to a Zeiss (Axioskop) light
Treatment tended to decrease TC modestly, although this was not statistically significant (Table). F1394 treatment had no effect on the plasma levels of HDL-C.

More detailed analyses of plasma lipoprotein CE content are summarized in Figures II and III in the online-only Data Supplement. There were no obvious differences in the pattern of fatty-acid-type esterified to cholesterol related to treatment in the different lipoprotein classes (Figure II in the online-only Data Supplement). Specifically, there was no difference in the percentage of CE that was cholesteryl oleate (thought to be particularly atherogenic) in any lipoprotein class (Figure III in the online-only Data Supplement).

**Effects of ACAT Inhibition on the Progression of Preexisting Atherosclerotic Lesion Size and Lipid Content**

After 14 weeks on WD, apoE−/− mice developed advanced atherosclerotic lesions in the aortic root containing necrotic lipid cores and cholesterol clefts, as shown in Figure 1A. WD feeding for 14 more weeks led to further progression of the lesion, as reflected by the ≈3-fold increase in size (Figure 1B) and the ≈8-fold increase in lipid content (Figure 1C). As shown (Figure 1A and 1B), F1394 treatment retarded the progression in lesion size by about 30% (P<0.05) and in lipid content, as determined histologically (Figure 1C), by ≈50% (P<0.05).

Changes in aortic lipid content were also measured chemically and the results are summarized in Figure 2. As shown, at the end of the 28-week study, aortic TC, FC, and CE contents were all statistically lower in the Treatment versus No Treatment group.

**Effects of ACAT Inhibition on Plaque Contents of Macrophages and Tissue Factor**

As shown in Figure 3A, macrophage marker Mac-2–positive areas were localized mainly to the subendothelial area in all experimental groups. Compared with the baseline group, over the next 14 weeks of WD feeding, without ACAT inhibition there was an increase in the Mac-2–positive area, which was prevented by F1394 treatment (Figure 3A and 3B).

Tissue factor (TF) content of plaques is considered to be the major culprit in the formation of the occlusive thrombus that follows plaque rupture. Although all 3 of the major cell types (endothelial cells, macrophages, and smooth muscle cells) can produce TF, we have found that the majority of it is associated with macrophages. Consistent with this association, as shown in Figure 4A and 4B, the distribution of TF staining and its quantitative changes were parallel to the corresponding data for Mac-2. In other words, without ACAT inhibition, TF content rose in plaques in the apoE−/− mice continued on the WD for an additional 14 weeks but were maintained at the pretreatment levels in the mice fed the WD containing F1394.

**Effects of ACAT Inhibition on Apoptosis, Plaque Necrosis, and Efferocytosis of Cells in Atherosclerotic Lesions**

Complete deficiency of ACAT1, the major macrophage ACAT isoform, has been shown to cause increased lesional FC, macrophage death, and tissue inflammation associated...
with increased atherosclerotic lesions. In studies using macrophage cell lines in which ACAT was inhibited (by Sandoz compound 58035) at the same time cells were loaded with cholesterol, it was shown that one basis for the macrophage cell death in ACAT1-deficient mice was the accumulation of FC in the ER membrane, triggering an ER-stress response that culminated in apoptosis.

Given that under the present experimental conditions the ACAT inhibitor actually decreased lesional FC, not increased it, we predicted that lesional macrophage apoptosis would not be increased. As shown in Figure 5A, TUNEL-positive areas were present in most animals from all experimental groups. As expected, the positive areas were close to the necrotic cores. Compared with the baseline group, with continued feeding of the WD, TUNEL staining increased as lesions further advanced (Figure 5B). As predicted by the decrease in lesional FC, partial ACAT inhibition was associated with decreased TUNEL staining (Figure 5B). The pattern of TUNEL data was independent of whether the area of staining or the percentage of nuclei positive for the staining (Figure 5C) was assessed. Although the decrease in apoptosis is perfectly consistent with the decrease in plaque FC, this finding indicates that the decrease in lesional macrophages cannot be explained by an increase in macrophage death. In addition, although there was an increase in the necrotic areas of the plaques associated with continued WD feeding, necrosis was not greater in the F1394-treated group relative to the untreated group; in fact, the trend was for a smaller increase with treatment (Figure 5D). There was also no difference in efferocytosis (the clearance of dead cells) in the plaques of mice treated or not treated with F1394 (Figure 6).

Effects of ACAT Inhibition Were Independent of Plasma Lipid Levels

As shown in the Table, ACAT inhibition resulted in a modest decrease in the plasma levels of non–HDL-C, as expected from its effects on intestinal cholesterol absorption and VLDL CE content. Nonetheless, the levels in the F1394 group remained high and in the range known to be highly atherogenic. To exclude that the effects of ACAT inhibition on lesion area or macrophage content were not indirect through changes in plasma cholesterol levels, as before, correlation analyses were performed. The Spearman r values for the correlations between plasma TC and the macrophage content and plaque area were 0.23 (P=0.24) and 0.20 (P=0.31), respectively, indicating that the effects of ACAT inhibition were independent of plasma lipid levels.

To further support the finding that plasma TC was not statistically related to any of the outcomes and does not account for the differences in them among the groups, analyses of covariance (ANCOVA) were performed to compare the outcomes among the 3 study groups (baseline, treatment, no treatment), adjusting for plasma TC. There were still significant differences in the outcome measures among the 3 groups, when
adjusting for TC. In other words, adjustment for plasma TC was not significantly associated with the outcome in any analysis. In a separate analysis in which we normalized for plaque size, the patterns we reported for changes in macrophage and tissue factor content in the treatment group were also not changed.

Discussion

In a previous report, we showed that partial inhibition of ACAT reduced the initiation and progression of early atherosclerosis in apoE−/− mice without evidence of obvious toxicity, in agreement with a recent report in which a specific ACAT2 inhibitor was used in these mice. In the present study, we have focused on a more clinically relevant scenario—the effects of partial ACAT inhibition on established atherosclerotic plaques that progressed beyond the foam cell stage. Compared with the baseline group, over the next 14 weeks, the plaque size increased, but the progression of increase was significantly retarded in the F1394 group. ACAT inhibition was also associated with beneficial changes in plaque composition. These included fewer macrophages and reductions in the content of tissue factor or neutral lipid in general, and TC, FC, and CE in particular, important factors in the pathophysiology of atherothrombosis. Furthermore, these effects were not associated with toxicity that was systemic (eg, normal weight gain and no dermal pathology) or in the plaques themselves, as assessed by the level of apoptosis, efferocytosis, or signs of necrosis.

Figure 2. Effects of Acyl-CoA:cholesterol acyltransferase (ACAT) inhibition on aortic free cholesterol (FC), cholesteryl ester (CE), and total cholesterol (TC) content. Aortic FC (A), CE (B), and TC (C) contents in baseline, untreated (No Treatment), and treated with F1394 ACAT inhibitor (Treatment) mouse aortas were measured by gas-liquid chromatography. Results expressed as µg FC, CE, or TC per g of aortic protein. Baseline, n=7; No Treatment, n=7; and Treatment, n=6. Data analyzed by Bonferroni Multiple Comparison test. Data are expressed as mean±SEM. Statistically significant differences (P<0.05, P<0.01, and P<0.001) are represented by *, **, and ***, respectively.

Figure 3. Effects of Acyl-CoA:cholesterol acyltransferase (ACAT) inhibition on lesion macrophage content. A, Representative sections stained for macrophages using the Mac2 and CD68 antibody were visualized at ×200. B, Bar graph represents measurements of stained areas for Mac2 and CD68. Baseline, n=9; No Treatment, n=12; and Treatment, n=12. Data analyzed by Bonferroni’s Multiple Comparison test. Data are expressed as mean±SEM. Statistically significant differences (P<0.05, P<0.01, and P<0.001) are represented by *, **, and ***, respectively.
The lack of an increase in apoptosis is perfectly consistent with the drug-induced decrease in lesional FC.

As expected, ACAT inhibition tended to have a modest impact on plasma cholesterol levels (Table), although it was not statistically significant. As in our previous study,5 there were no statistically significant correlations between the major findings and plasma cholesterol levels. As before, even in the F1394 group, plasma cholesterol levels (>1000 mg/dL)
Counterbalancing the potential benefits of ACAT inhibition is the literature that partial or complete deficiency of ACAT activity is proatherogenic. For example, mice with complete deficiency of macrophage ACAT (ACAT1−/− mice) have evidence of large, inflamed plaques and skin pathology, attributed to the deposition of crystals of FC. Although it is tempting to speculate that the difference between this study and the beneficial outcomes in many preclinical inhibitor studies was the degree of toxicity, 2 clinical studies52,53 also showed that ACAT inhibition did not reduce plaque volume as measured by intravascular ultrasound, although intravascular ultrasound would not have detected the changes in plaque composition as the histological assays we used can. As recently reviewed,54 a plausible explanation for these apparent clinical failures includes the accumulation of FC in macrophages and foam cells, which, in addition to the formation of toxic cholesterol crystals, can also lead to ER stress, activation of the inflammasome, apoptosis, and impaired efferocytosis of dead cells.5,17,39,55-57

The mechanism(s) for the apparent discrepancies between the present results and the clinical trial data will require further study. A key feature of the study here was a decrease in lesional FC that is thought to be a key determinant of advanced lesional macrophage death and plaque necrosis. Thus, in settings where plaque FC increases, such as in the course of normal advanced plaque progression or in the setting of complete ACAT1 inhibition, advanced lesional macrophage apoptosis and plaque necrosis ensue. Accordingly, we propose that under conditions of partial ACAT inhibition by the drug used in this study, either enough HDL particles were available to prevent surpassing a critical threshold level of FC in the macrophages and foam cells, or the residual level of ACAT activity (estimated to be ≈50%)5 was sufficiently protective.

In summary, we have shown that when mice with established plaques are treated with ACAT inhibitor F1394, there are major beneficial results characterized by slower plaque progression and decreased contents of macrophage foam cells, lipids, and tissue factor. Taking into account the previous animal and clinical data, these results argue that the inhibition of ACAT is still a viable clinical target to retard plaque progression, but that the dosing may be the critical issue. Too much or too little inhibition is likely to be undesirable because of cellular toxicity or lack of efficacy, respectively. Although this is an important practical issue to resolve, until current imaging techniques are sufficiently sensitive to changes in plaque composition, important potential benefits of ACAT inhibition that are effected through changes in plaque composition may go undetected, making the determination of efficacy difficult to assess.

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Disclosures
None.
References


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Supp. Figure I: Effect of F1394 ACAT inhibitor on ACAT 2 activity in the liver. ACAT 2 activity was assayed in microsomes isolated from liver samples of mice untreated (“No Treatment”) and treated (“Treatment”) with F1394 ACAT inhibitor. Activity is reported as nmol $[^{14}C]$oleoyl-CoA per mg microsomal protein per min. N=3 for each group. Inhibition of ACAT 2 by F1394 was statistically significant (P < 0.05) as analyzed by two-tailed t-test. Data are expressed as Mean ± SEM.
Supp. Figure II: Effects of ACAT inhibition on plasma cholesteryl ester content. Fatty acid methyl ester (FAME) analyses were performed on HDL-CE, LDL-CE, and VLDL-CE of mice (N=2 of 2 pooled plasma samples) not treated (A) and treated (B) with F1394 ACAT inhibitor. The bars represent the mean (± SEM) percentages of saturated, monounsaturated, and polyunsaturated CE in HDL, LDL, and VLDL for untreated and treated mice; the inset indicates which pattern corresponds to which type of CE.
Supp. Figure III: Effects of ACAT inhibition on plasma cholesteryl oleate content. Bars represent cholesteryl oleate content as a percentage of CE in HDL, LDL, and VLDL of mice (N=2 of 2 pooled plasma samples) not treated (“No Treatment”) and treated (“Treatment”) with F1394 ACAT inhibitor. There was no significant change in plasma cholesteryl oleate content between the two groups as analyzed by two-tailed T-test. Data are expressed as Mean ± SEM.
Supp Figure IV. Effects of ACAT inhibition on vascular cell adhesion molecule-1 content. A) Representative sections stained for VCAM-1 visualized at 40X. Arrows indicate endothelial cell layer; note the increased staining in the no treatment section. B) Bar graph represents rated intensity of VCAM-1 endothelial cell layer staining. Four blinded observers scored the intensity of VCAM-1 staining in the endothelium from four separate sections from each mouse on a scale of 1 to 5: 1 representing the least intense, 5 representing the most. The untreated “No Treatment” and treated “Treatment” (N=5 mice for each group) endothelial stain intensities were 3.50±0.32 and 2.75±0.28 (mean ± SEM), respectively. ACAT inhibition decreased VCAM-1 content in a statistically significant manner (P < 0.05), as analyzed by one-tailed t-test.