Acyl-CoA:Cholesterol Acyltransferase Inhibition Reduces Atherosclerosis in Apolipoprotein E–Deficient Mice

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Background—Acyl-CoA:cholesterol acyltransferase (ACAT) converts cholesterol to cholesteryl esters. The form of ACAT in macrophages, ACAT1, contributes to foam cell formation in the arterial wall and the development of atherosclerosis. Recent studies in a mouse model of atherosclerosis (the apolipoprotein E [apoE]–deficient mouse), however, have suggested that complete deficiency of ACAT1 activity is not antiatherogenic, in part because of toxicity resulting from adverse effects on tissue cholesterol homeostasis. We have tested whether partial inhibition of ACAT1 and ACAT2 (expressed in liver and intestine) activities reduces atherosclerosis development in apoE-deficient mice and avoids toxicity.

Methods and Results—ApoE-deficient mice were maintained for 17 weeks on a Western-type diet without (control) or with the ACAT inhibitor F-1394 (effective against ACAT1 and ACAT2) at doses of either 300 (low) or 900 (high) mg/kg. Intimal lesion area at the aortic sinus in controls was 0.69±0.06 mm². F-1394 treatment significantly decreased lesional area by 39% (low) or 45% (high). F-1394 treatment also reduced lesional immunostaining for macrophages by 61% (low) or 83% (high). En face analysis showed that surface lipid staining in control aortas was 20.0±2.8%; F-1394 treatment reduced this by 46% (low) or 62% (high). There were no obvious signs of systemic or vessel wall toxicity associated with F-1394 treatment.

Conclusions—Partial ACAT inhibition by F-1394 had antiatherogenic effects in apoE-deficient mice that were achieved without obvious toxicity. Partial inhibition may have therapeutic potential in the clinical treatment of atherosclerosis. (Circulation. 2001;103:2604-2609.)

Key Words: aorta ▪ cholesterol ▪ plaque

Atherosclerosis leads to coronary heart disease (CHD). Although statin drugs are powerful weapons against atherosclerosis, CHD risk remains high, making it desirable to develop additional therapeutic approaches, including the inhibition of acyl-CoA:cholesterol acyltransferase (ACAT). This enzyme catalyzes cholesterol esterification and plays important roles in lipoprotein assembly, dietary cholesterol absorption, and intracellular cholesterol metabolism. Most relevant to CHD is that in the arterial wall, cholesteryl ester produced by the form of ACAT known as ACAT1 can accumulate in macrophages (Mø) and smooth muscle cells (SMCs) to produce foam cells, leading to plaque initiation and atherosclerotic progression.

Given the function of ACAT1 and the limited evidence from cell and animal studies, it has been proposed that inhibition of ACAT1 would have antiatherosclerotic effects. Yet, it has been reported recently that complete deficiency of ACAT1, produced by gene-targeting techniques, did not prevent lesion development in 2 hyperlipidemic mouse models of human atherosclerosis (apolipoprotein E–deficient [apoEKO] or LDL receptor–deficient mice). These results were most striking when the animals were fed a fat and cholesterol-enriched diet to further elevate plasma lipid levels and thereby provide a maximal atherogenic stimulus. Tissue cholesterol homeostasis was adversely altered, leading to accumulated cholesterol crystals, cellular toxicity, and the development of atherosclerotic lesions with abnormal composition. These results raise the important question of whether partial inhibition of ACAT1 activity would retard atherosclerosis development while avoiding adverse tissue effects because of a less severe perturbation of cholesterol homeostasis.

To address this question, we treated apoEKO mice with compound F-1394 ((1s,2s)-2-[3-(2,2-dimethylpropyl)-3-nonylureido]cyclohexane-1-yl 3-[(4R)-N-(2,2,5,5-tetramethyl-1,3-dioxane-4-carbonyl)amino]propionate), a highly specific and potent inhibitor of both forms of ACAT (ACAT1 and ACAT2, the latter expressed in liver and intestine) in vitro and in vivo. The inhibition of ACAT2 may also be antiatherogenic by decreasing the absorption of cholesterol in the intestine, reducing the production of...
hepatic VLDL particles, or decreasing VLDL cholesteryl ester content (for recent reviews, see Brewer and Joyce et al).

ApoEKO mice have been used previously to test many potential antiatherosclerosis factors (eg, Bourassa et al). As will be presented, partial inhibition of ACAT by F-1394 treatment in apoEKO mice fed a fat and cholesterol-enriched diet resulted in decreases in atherosclerotic lesion size, lesional Mø content, and aortic lipid content, but no signs of systemic or arterial wall toxicity. These exciting results suggest that partial inhibition of ACAT has promise as a safe and effective clinical treatment for atherosclerosis.

Methods

Compound F-1394 was supplied by Fujirebio Inc (Tokyo, Japan). As reported previously, it is potent as an inhibitor of ACAT activity in cells and tissues containing ACAT1 or ACAT2, with IC values of ~10 for either form. The comparable inhibitory activity of F-1394 has recently been confirmed with recombinant human ACAT1 and ACAT2 reconstituted into microsomes (J.K., unpublished data, 1999).

Animals and Diets

All procedures were approved by the Animal Care Committee. Thirty apoEKO mice (male and female, C57BL strain; Jackson Laboratory, Bar Harbor, Maine) were fed a regular chow diet (Purina) until 12 weeks of age. The Western-type diet, which contained (wt/wt) 21% fat, 0.15% cholesterol, and 19.5% casein, was purchased from Dyets Inc and used as supplied or supplemented with F-1394 (300-mg/kg and 900-mg/kg diet). The F-1394 content of the F-1394/kg food or “high F-1394” group, as were weight gains during the treatment period (data not shown). The dermatologic changes in ACAT1 knockout mice (alopecia and dermal hypertrophy) were not observed in either F-1394 group.

Based on the weight of unconsumed food pellets, the daily dose of F-1394 in the low F-1394 group was 76.6 ± 3.9 mg/kg mouse. Exact food consumption in the high F-1394 group could not be measured because the food pellets easily crumbled. Given the comparable weight gains among the 3 groups, however, an equivalent amount of food was likely consumed in the high F-1394 group, making the estimated daily dose of F-1394 in this group ~230 mg/kg mouse.

Results for each group (n=10 per group) are expressed as mean±SEM. Statistical differences among groups were determined by 1-way ANOVA with Bonferroni multiple comparison tests as indicated. Correlation between parameters was determined by linear regression analysis and Spearman correlation. All statistical analyses were performed with GraphPad Prism software (GraphPad Software, Inc).

Statistical Analysis

Results for each group (n=10 per group) are expressed as mean±SEM. Statistical differences among groups were determined by 1-way ANOVA with Bonferroni multiple comparison tests as indicated. Correlation between parameters was determined by linear regression analysis and Spearman correlation. All statistical analyses were performed with GraphPad Prism software (GraphPad Software, Inc).

Cholesterol Measurement

Plasma total cholesterol (TC) was measured (at 11, 20, and 28 weeks of age) with a commercial kit (Sigma Diagnostics).

Data are reported as mean±SEM. Statistical differences among groups were determined by 1-way ANOVA with Bonferroni multiple comparison tests as indicated. Correlation between parameters was determined by linear regression analysis and Spearman correlation. All statistical analyses were performed with GraphPad Prism software (GraphPad Software, Inc).
appeared to be an increase in SMC-appearing cells (an example of one is indicated by the arrow in Figure 2D).

F-1394 treatment was associated with decreased Mø content of the neointima as assessed visually (Figure 3) and by image analysis of sections stained with an anti–MOMA-2 antibody (Figure 1B). Abundant Mø (stained brown) were observed throughout the neointima in the control mouse (Figures 3A and 3C; different magnifications of same section). In contrast, in the high F-1394 group (Figures 3B and 3D; different magnifications of the same section), fewer Mø were found, and they were predominantly subendothelial (eg, arrow in Figure 3D). The percentage of the neointima that stained positive for MOMA-2 declined from \( \approx 18\% \) in the sample from the control mouse to \( \approx 2\% \) in the sample from the F-1394 mouse. Overall, the average area of MOMA-2 staining (relative to the control group) was significantly reduced by 61\% and 83\% in the low and high F-1394 groups, respectively (Figure 1B).

Because instability of plaques has been related to Mø enrichment of their shoulder regions,\(^7\) we examined these areas for effects of F-1394. Shoulder regions of aortic sinus lesions are shown in Figure 4. In serial sections (Figure 4A [H&E staining] and Figure 4B [MOMA-2 staining]) from a control mouse, many Mø-derived foam cells (lipid-engorged MOMA-2–positive [brown stained] cells in Figure 4B) are visible and are overlaid by endothelial cells (the layer of endothelial cells is indicated by arrows in Figures 4A and 4B). In contrast, in the shoulder region of a lesion from a F-1394–treated mouse, there is a striking reduction in Mø content (Figure 4C, H&E; Figure 4D, MOMA-2 staining).

### En Face Lipid Staining

In the control group, the area of the thoracic and abdominal aorta stained by Sudan IV was 20.0\%±2.8\%. In both the low and high F-1394 groups, there were significant decreases in the area stained (Figure 5); relative to the control group, the reductions were 46\% (low group) and 62\% (high group), consistent with the decreased lesion areas and Mø contents noted above.

### Correlations of the Effects of F-1394 With Lesion Parameters

Linear regression analyses were performed to test whether the effects of F-1394 on plasma TC levels could explain the reduction in atherosclerosis assessed by either morphometric or en face analysis. As summarized in the Table, the neointimal lesion area in the aortic sinus significantly correlated with both the Mø-positive area of the lesion and the surface lipid staining of the aorta. There was also a significant correlation between surface staining and the Mø-positive area. In contrast, as shown in the Table, the plasma TC level did not significantly correlate with the neointimal area, Mø-positive area, or surface lipid staining. Because of the clustering of plasma TC values in the hyperlipidemic range,
which may have impaired the detection of significant correlations by linear regression, we also examined the relationships between TC values and lesional parameters by Spearman correlation testing. Again, there were no statistically significant correlations.

Discussion
The major finding from our studies is that in apoEKO mice, a standard model of human atherosclerosis, partial ACAT inhibition by F-1394 results in beneficial quantitative and qualitative changes in arterial lesions. These include a smaller intimal area, reduced Mø content, less neutral lipid content of the aorta, and a more stable appearance of the “shoulder region.”

These changes could not be statistically attributed to a hypolipidemic effect, although depending on the length of treatment and the dose, plasma TC levels were decreased by F-1394. This decrease was probably due in part to lower cholesterol absorption in mice treated with F-1394, based on our previous data in other species.10,11,18,19 Another factor potentially affecting plasma lipoproteins is hepatic ACAT inhibition by F-1394,12 which could favorably change VLDL particle number or composition.13,14

The persistent and severe hypercholesterolemia (>1000 mg/dL) in the F-1394 treatment groups and the lack of correlation between the TC level and lesional parameters, however, imply that there was a direct effect on ACAT activity in the arterial wall (see also Matsuo et al6 and Bocan

Figure 3. Mø content of atherosclerotic lesions in aortic sinus of apoEKO mice. Mice were treated as in Figure 1. Representative sections from control mice (A and C) and F-1394–treated (300 mg/kg diet) mice (B and D) immunostained with antibody to Mø marker MOMA-2 are shown at low (×12.5) and higher (×100) magnification. Brown indicates positive immunostaining. Arrow and asterisk in D indicate examples of subendothelial Mø and cholesterol clefts, respectively.

Figure 4. Photomicrographs of typical shoulder regions of atherosclerotic lesions in aortic sinus of apoEKO mice. Mice were treated as in Figure 1. Sections were stained with either H&E (A and C) or anti–MOMA-2 antibody (brown staining; B and D). Sections in A and B are from control mouse, and C and D are from F-1394–treated (300 mg/kg diet) mouse. Arrows indicate endothelial layer over neointimal lesion in control mouse. All photomicrographs are at ×200 magnification.
The clustering of TC values in the hypercholesterolemic range, however, may have impaired the detection of significant correlations between plasma TC levels and the other parameters. Two additional pieces of information, therefore, may be helpful in placing the statistical results in context. The first is that despite the generally elevated mean plasma TC values, in the combined data set, the values varied from 614 to 2365 mg/dL, approximately a 4-fold difference, a range likely to have been wide enough to reveal strong correlations. As alluded to just above, however, there could still be subtle effects from ACAT inhibition on hepatic and intestinal lipoproteins that would not be detectable in the regression or Spearman analysis. Second, F-1394 is also effective in apoEKO mice maintained on a chow diet (J.K. and E.A.F., unpublished data). As noted, we were particularly interested in testing F-1394 under the dietary conditions in which ACAT1 deficiency was most toxic; hence, the Western-type diet was used. Preliminary data from a study in progress, using the same design as in the present study except that chow was used as the control diet, revealed that as expected, plasma TC levels at the end of the treatment period were lower than with the Western-type diet (chow 523 ± 76 mg/dL, low F-1394 422 ± 50 mg/dL, and high F-1394 545 ± 38 mg/dL; n = 6/group), but there was no decrease (versus control) associated with F-1394 treatment at the higher dose. Consistent with these lower TC values, mean intimal lesion areas in all groups were also less than in the present study (eg, control group mean = 0.20 ± 0.04 mm²; compare to Figure 1A), but treatment with either low- or high-dose F-1394 significantly reduced lesion area by ~54% (control versus either low or high F-1934 group, P < 0.02; low versus high F-1394, P = NS).

Taken together with the results from the present study, these data support the contention that a major effect of F-1394 is the reduced esterification of cholesterol in the arterial wall. In spite of the statistical analyses, however, we cannot exclude, especially in mice consuming the fat and cholesterol-enriched diets, the augmentation of effects of F-1394 on the arterial wall by the modification of plasma lipoprotein levels or properties secondary to the inhibition of hepatic and intestinal ACAT activities. In fact, the likelihood of this is perhaps best suggested in the present study by the larger decreases in lesional Mø and lipid contents in the high-dose F-1394 group, which also had the greatest lowering of plasma TC levels.

It is notable that the antiatherosclerosis effects of ACAT inhibition were obtained without evidence of systemic or arterial wall toxicity, because it has been speculated that ACAT inhibition in vivo may not be beneficial and may even result in some detrimental consequences. Indeed, in the ACAT1/ApoE double-knockout mouse model, there developed large atherosclerotic lesions with a number of unusual morphological features thought to be from major changes in tissue cholesterol homeostasis associated with the complete absence of ACAT1 activity. In addition, the animals exhibited dermatopathology from accumulated crystals of free cholesterol.

In contrast, F-1394 treatment clearly reduced the size of the lesions without causing any dermal changes, presumably because ACAT inhibition was partial. Although cholesterol clefts (which contain cholesterol crystals) were visible in some of the arterial lesions of F-1394–treated mice (eg, asterisk in Figure 3D), there was no evidence of inflammation of the type found in ACAT1/ApoE double-knockout mice. The presence of cholesterol clefts cannot be attributed to F-1394 treatment, because they also appear in the lesions of apoEKO mice fed a Western-type diet (eg, asterisk in Figure 2C). Thus, clefts most likely are a general consequence of the extremely high plasma TC levels (1000 to 2000 mg/dL). Additional evidence in support of the low toxicity of F-1394 has been obtained in several species of animals, such as rats, rabbits, dogs, and monkeys (toxicology group, Fujirebio Inc, unpublished data, 1995 to 1998). One explanation for the decrease in lesional area immunostained by antisera against Mø markers in animals genetically (eg, Accad et al) or pharmacologically manipulated to have reduced ACAT activity is that fewer Mø become “engorged” by cholesteryl ester and remain relatively small. It is also possible that Mø number in the lesions was reduced because the accumulation of unesterified cholesterol promoted Mø apoptosis or necrosis. We do not favor a predominant effect of ACAT inhibition by F-1394 on Mø necrosis or apoptosis, however, because it blocked foam cell formation in vitro without any increase in cytotoxicity (J.K., E.A.F., unpublished data, 1998).

Correlations Among Lesion and Lipid Parameters Based on Combined Data From All Groups

<table>
<thead>
<tr>
<th>Parameter</th>
<th>r</th>
<th>P</th>
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<tr>
<td>Neointimal area</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Vs plasma TC</td>
<td>0.116</td>
<td>0.546</td>
</tr>
<tr>
<td>Vs Mø content</td>
<td>0.574</td>
<td>0.002</td>
</tr>
<tr>
<td>Vs en face lipid staining</td>
<td>0.636</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Mø content</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Vs plasma TC</td>
<td>0.295</td>
<td>0.114</td>
</tr>
<tr>
<td>Vs en face lipid staining</td>
<td>0.639</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>En face lipid staining</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Vs plasma TC</td>
<td>0.297</td>
<td>0.118</td>
</tr>
</tbody>
</table>

Combined data from all mice were analyzed by linear regression analysis; correlation coefficients (r) and P values are shown.
The reduction of MOMA-2 immunostaining in the shoulder regions of lesions in F-1394–treated mice (Figure 4D) would be consistent with plaque stabilization.17,23,24 Further support for this is a relative increase in the content of SMCs and extracellular matrix in lesions in the treated mice (Figures 1 through 4). Plaque stabilization may also result from aggressive lowering of plasma LDL levels (eg, Shepherd et al25), implying that there may be additive benefits of hypolipidemic and anti-ACAT therapies.

In summary, in apoEKO mice, partial inhibition of ACAT by F-1394 led to a number of beneficial qualitative and quantitative changes in atherosclerotic lesions without obvious systemic or local toxicity. These results imply a similar strategy may have therapeutic promise in patients at high risk for primary or secondary CHD events resulting from atherosclerosis.

Acknowledgments

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References

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