Comparison of CI-976, an ACAT Inhibitor, and Selected Lipid-Lowering Agents for Antiatherosclerotic Activity in Iliac–Femoral and Thoracic Aortic Lesions

A Biochemical, Morphological, and Morphometric Evaluation

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Due to the potential importance of acyl-coenzyme A: cholesterol O-acyltransferase (ACAT) in the generation of lipid-filled macrophages, the ACAT inhibitor CI-976 (2,2-dimethyl-N-(2,4,6-trimethoxyphenyl)dodecanamide) was evaluated relative to selected lipid-lowering agents for their effect on atherosclerotic lesion regression and progression. Atherosclerotic lesions comparable in composition to human fatty streaks were induced by chronic endothelial denudation in the iliac-femoral artery of hypercholesterolemic New Zealand White rabbits before intervention, while naturally occurring fatty streaks developed in the thoracic aorta. CI-976 administered in a hypercholesterolemic diet at a dose that did not lower plasma cholesterol prevented the accumulation of monocytes–macrophages within the preestablished iliac–femoral lesion and reduced the foam cell area by 27–29% relative to the initiation of intervention. CI-976 also blunted the development of thoracic aortic fatty streak-like lesions and decreased the cholesteryl ester enrichment by 46%. CI-976 had no effect on plasma triglycerides and, more importantly, had no effect or decreased liver, iliac–femoral, and thoracic aortic free cholesterol content. Dietary intervention alone increased monocyte–macrophage involvement in the iliac–femoral lesion despite reductions in plasma, liver, and thoracic aortic cholesterol content. Conventional lipid-lowering therapy such as cholestyramine or cholestyramine/niacin required substantial decreases in plasma cholesterol levels to achieve comparable vascular changes. We conclude that inhibition of ACAT within the arterial wall by the potent and specific ACAT inhibitor CI-976, even in the absence of plasma cholesterol lowering, can result in the inhibition of atherosclerotic lesion progression and can enhance regression. (Arteriosclerosis and Thrombosis 1991;11:1830–1843)

Acyl-coenzyme A: cholesterol O-acyltransferase (EC 2.3.1.26; ACAT), the primary enzyme responsible for the esterification of cholesterol in all mammalian cells, has been implicated as a key enzyme involved in cholesterol absorption, very low density lipoprotein secretion, and the formation of lipid-filled macrophages and smooth muscle cells (SMCs). In addition, monocyte–macrophage foam cells have been shown to be integral components of human and animal atherosclerosis. In humans, both fatty streaks and fibrous plaques are characterized by the presence of lipid-filled macrophages; however, in fibrous plaques the appearance of abundant extracellular lipid and lipid-filled SMCs is also noted. Several investigators, using monoclonal antibodies to monocytes–macrophages, have shown that monocytes–macrophages in animals are involved in the initiation and development of the fatty streak and the conversion to the fibrous plaque. Since ACAT is responsible for the esterification and storage of cholesterol in the macrophage, one might hypothesize that inhibition of arterial wall ACAT may prevent the formation of the macrophage-enriched fatty streak and the development of the clinically significant fibrous plaque.

Several investigators, using either specific or non-specific inhibitors of ACAT, have examined the...
The effect of ACAT inhibition on aortic cholesteryl ester content and lesion severity. These reports suggest that arterial ACAT inhibition may beneficially affect atherosclerotic lesion development. However, reductions in plasma cholesterol and/or specificity of the inhibitor would prevent one from unequivocally attributing the changes in arterial cholesteryl ester content to ACAT inhibition alone.

In the present study, the specific and potent ACAT inhibitor CI-976 (2,2-dimethyl-N-(2,4,6-trimethoxyphenyl)dodecanamide) was evaluated for its effect on atherosclerotic lesion progression and regression. We have previously reported that CI-976 inhibited ACAT in rabbit intestinal microsomes and mouse peritoneal macrophages by 50% at 0.075 μM and 0.62 μM, respectively. CI-976 was also shown to inhibit cholesterol absorption in the rat, to lower plasma total cholesterol, and to elevate high density lipoprotein cholesterol in cholesterol-fed rats. Field and colleagues have also reported that CI-976 (PD128042) inhibits the ACAT activity of CaCo-2 cells, an intestinal cell line, by 50% at 0.51 μM. The present study is unique in that CI-976 was evaluated in a rabbit model of atherosclerosis with preestablished iliac-femoral atherosclerosis. The atherosclerotic lesions resemble human fatty streaks and fibro-lipid lesions in their cellular and extracellular composition, that is, the presence of lipid-filled monocytes—macrophages, SMCs, extracellular lipid, and connective tissue deposition. In this same animal model, it was feasible to evaluate the effect of agents on the progression of lesions within the thoracic aorta and to examine the regression of preestablished iliac–femoral lesions.

Thus, the aim of the present study was fourfold: first, to evaluate the effect of ACAT inhibition on atherosclerotic lesion development at two dose levels; one that does not lower plasma cholesterol and one that does decrease plasma cholesterol; second, to examine the effect of pharmacological intervention in a model that is normally nonresponsive to dietary-induced lesion regression; third, to compare the changes observed with CI-976 to dietary modification, cholestyramine, niacin, or cholestyramine plus niacin treatment; and finally, to provide a biochemical, morphological, and morphometric evaluation of the antiatherosclerotic properties of CI-976 and selected lipid-lowering therapies.

Methods

Experimental Design

Male New Zealand White rabbits (Kuiper Farms, Gary, Ind.) weighing 1.2–1.5 kg were meal-fed a chow diet (Purina, No. 5321) supplemented with 0.5% cholesterol, 3% peanut oil, and 3% coconut oil between 7 and 9 AM daily for a total of 17 weeks. The dietary regimen consisted of feeding 40 g for the first week, 50 g for the next 4 weeks, 60 g for another 4 weeks, and 70 g for the final 8 weeks. After 1 week of diet initiation, a chronic endothelial injury was induced in the abdominal and femoral arteries by surgically inserting a sterile, indwelling, 18-cm nylon monofilament with a diameter of 200 μm into the lumen of the right femoral artery. As reported previously, a fibrofoamy, lipid-rich iliac–femoral lesion had developed 8 weeks after injury in 99% of the animals. The animals were randomized based on their plasma cholesterol values, which were determined in animals 24 hours after their daily meal. The groups were chosen when the F ratio determined by analysis of variance was <0.5 and the percent standard error of the mean difference was <25% (ALOCATE Software, Elsevier). The animals were subsequently divided into control and drug-treatment groups. The results reported here are based on a compilation of a number of experiments, and the final group size ranged from seven to 31 animals. The time-zero group (number of animals [n]=22) was used to assess the extent of lesion development in the iliac–femoral artery and the thoracic aorta at the initiation of diet or drug intervention. As noted in Figure 1, the effect of diet and pharmacological intervention on the regression of preestablished atherosclerotic lesions was studied in the iliac–femoral artery. In this study, regression was defined as a reduction in the lesion lipid content, lesion area, or monocyte–macrophage foam cell area. The effect of therapy on the progression of naturally occurring lesions was assessed in the thoracic aorta. Atherosclerotic lesion progression was characterized by an increase in lipid content or thoracic aortic lesion coverage relative to the beginning therapy, that is, at time zero, 9 weeks after diet initiation. A group of animals termed the progression control (n=29) was maintained on the cholesterol plus fat diet for the remaining 8 weeks. Animals in the regression control group (n=31) had their diet switched from the cholesterol plus fat-containing diet to a normal chow, low-cholesterol diet. The pharmacological agents noted below were administered as a diet admixture in the cholesterol plus fat-containing diet for the final 8 weeks. The specific and potent ACAT inhibitor CI-976, whose synthesis was previously described, was dosed at 5 mg/kg body wt or 25 mg/kg body wt (n=7 and 14, respectively). In addition, separate groups of animals were dosed with either niacin (n=8; Sigma Chemical Co., St. Louis, Mo.) at 200 mg/kg body wt, cholestyramine (n=7; 500 mg/kg body wt; Purolite, Philadelphia, Pa.), or cholestyramine/niacin (n=7; 500:200 mg/kg body wt).

Chemical Methods

Plasma cholesterol and triglyceride levels were measured enzymatically throughout the study on an Abbott VP Series II Bichromatic Analyzer (Abbott, Chicago, Ill.) with the Boehringer-Mannheim total cholesterol reagent (Indianapolis, Ind.) and the Abbott triglyceride reagent. The lipid measurements were made on a monthly basis before drug administration and biweekly after intervention. Liver total and free cholesterol contents were also measured.

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Liver samples were homogenized, and the lipids were extracted in a 20-fold excess of isopropyl alcohol. Aliquots of the isopropyl extracts were assayed for their total and free cholesterol contents by a modification of the enzymatic, colorimetric method of Cooper and coworkers, which has been previously described.

The descending thoracic aorta and segments of iliac–femoral arterial lesions adjacent to those collected for histological evaluation were assayed for their total cholesterol, cholesteryl ester, free cholesterol, and phospholipid content. A vessel segment extending from the aortic–iliac bifurcation to the last branch of the femoral artery before the total occlusion induced by the secured nylon mononlament was taken for lipid analysis and histological evaluation. The first 1-cm segment extending from the aortic–iliac bifurcation was used for histological evaluation, and the remaining 3-cm segment was extracted for lipid quantification. The adventitia and media were stripped from the vessels along a cleavage plane created by the internal elastic lamina, and intimal preparations were minced. The lipids were extracted in chloroform/methanol (2:1) by the procedure of Folch et al. The extracts were washed with 0.74% KCl. The organic layer was collected, dried under nitrogen, and redissolved in 50–250 μl chloroform. The resultant samples were stored at −20°C until analyzed. The lipid composition of the iliac–femoral lesions and thoracic aortas was measured with an Iatroscan TH-10 Mark IV TLC-FID analyzer (RSS Inc., Costa Mesa, Calif.) attached to an HP3390A integrator (Hewlett-Packard Co., Palo Alto, Calif.).

Cholesteryl ester and free cholesterol were measured directly, while the total phospholipid content was determined by summation of the various phospholipid subclasses. The iliac–femoral arterial lesion lipid composition was analyzed in two ways. The absolute lipid mass, that is, an indicator of lipid loading of the blood vessel, was determined, and the relative lipid weight percentage, that is, an indicator of lesion type relative to human atherosclerotic lesions, was calculated.

Cytochemical Methods

For histological evaluation of the iliac–femoral lesions, a segment of the iliac–femoral artery 1 cm distal to the aortic–iliac bifurcation was embedded in OCT compound (Miles, Elkhart, Ind.), flash-frozen on a carbon dioxide-chilled metal plate, and stored at −20°C until sectioned. The flash-frozen samples of the iliac–femoral lesions were sectioned at 10 μm on a Reichert Histostat (Fisher, Pittsburgh, Pa.). The frozen lesion sections were stained with oil red O in 60% isopropyl alcohol to demonstrate the distribution of lipid. In addition, tissue sections were stained with hematoxylin and eosin for general morphology and with toluidine blue and Verhoeff's elastica stain for the connective tissue elements. The α-naphthyl acetate method for nonspecific acid esterase was used to stain selectively for cells with a high content of lysosomal enzymes and were thereby identified as monocytes–macrophages. Watanabe and coworkers have previously reported that nonspecific esterase reaction prod-
uct and monoclonal antibodies to rabbit macrophages colocalized to the same population of cells within the atherosclerotic lesions.

**Morphometric Methods**

Tissue sections stained specifically for monocytes-macrophages by the α-naphthyl acetate procedure were used for the quantification of macrophage and lesion areas. Images of the stained specimens were traced with the aid of a camera lucida attached to a Leitz Diaplan Microscope (W. Nushbaum Inc., McHenry, Ill.). The whole lesion was defined as the area between the lumen and the internal elastic lamina, which was identified as a translucent, wavy line when viewed by phase microscopy. The monocytes-macrophages were identified as cells that expressed positive esterase staining, were birefringent under polarized light, and stained positively with oil red O.

Intramacrophage lipid stained with oil red O was characterized by a globular staining appearance overlaying and surrounding a nucleus. A total of three sections that were spaced approximately 200 μm apart were traced through the camera lucida at a magnification of x40. Groups of macrophages and individual cells were recorded. The specimens were quantified by tracing the images on a Kurta digitizing pad (Kurta, Phoenix, Ariz.) attached to an IBM PS/2 Model 50 computer. Lesion and macrophage areas were determined with SIGMA SCAN software (Jandel Scientific, Corte Madera, Calif.). The lesion area was defined as the cross-sectional area delineated as the lumen-lesion edge to the internal elastic lamina. The total monocyte-macrophage foam cell area was the sum of stained areas identified within a particular specimen. The mean lesion and macrophage areas were determined for each specimen, and the average per group was calculated based on the mean specimen area.

The percentage of the thoracic aorta covered by atherosclerotic lesions was also determined. Photographs of the thoracic aorta from the aortic valve to the diaphragm were obtained at necropsy. The individual specimens were enlarged to 10 times their original size, and photographic prints were prepared. For each specimen, the location of the first to seventh intercostal ostia was marked to match the sample selected for biochemical analysis. The photograph was affixed to the digitizing pad, and the area of the thoracic aorta between the first and seventh intercostal ostia was traced. The area of the aortic lesions was also determined in subsequent tracings. An atherosclerotic lesion was defined as any flat or raised image with a defined border relative to the surrounding vessel. The percent lesion coverage was calculated as the sum of the total lesion area divided by the aortic area between the first and seventh intercostal ostia. The average percent lesion area was calculated per group.

**Statistical Analyses**

Statistical analysis of the biochemical and morphometric data was performed with an analysis of variance procedure followed by a two-tailed Student’s t test for individual comparisons. The level of significance, the probability value, was established for statistical comparisons made relative to the progression, regression, and time-zero control. To ensure an unbiased result, the data were collected in a double-blinded fashion. The specimens were ascribed to their respective treatment group after the morphometric measurements were obtained.

**Results**

**Plasma and Liver Lipid Content**

Plasma cholesterol levels rose sevenfold to 12-fold relative to the initiation of the study during the first 9 weeks of cholesterol feeding (Figure 2). At this time when diet or drug intervention began, that is, time zero, plasma cholesterol levels ranged from 925 to 1,489 mg/dl; however, they were not significantly different among the various treatment groups at p<0.05. Plasma cholesterol levels of the progression control (animals maintained on the cholesterol diet without drug intervention) rose 11% relative to time zero. Dietary intervention decreased plasma cholesterol 71%. CI-976 at 25 mg/kg significantly lowered
plasma cholesterol 56%, while at 5 mg/kg no significant reduction was noted. Cholestyramine and the cholestyramine/niacin combination markedly lowered plasma cholesterol levels by 74% and 96%, respectively. Niacin alone had no effect on plasma cholesterol. When the data were compared relative to the progression control, all treatments except CI-976 at 5 mg/kg significantly lowered plasma cholesterol 44–97% (Figure 2). During the course of the study, plasma triglycerides were unchanged with any treatment, that is, they remained at approximately 100 mg/dl throughout the study. No differences in body weight gain or food consumption were noted among any of the groups.

Total cholesterol content in the liver at time zero increased ninefold relative to the initiation of the study. In the progression control, liver total cholesterol rose 61% relative to time zero (Table 1). Dietary intervention significantly lowered liver total cholesterol content 50%, while CI-976 at either dose had no effect on the cholesterol content. However, at the 25 mg/kg dose, liver free cholesterol content was significantly reduced 30%. Cholestyramine and the cholestyramine/niacin combination decreased liver total cholesterol content 71% and 90%, respectively, and lowered free cholesterol content 58% and 81%, respectively. Niacin treatment significantly elevated liver total cholesterol 44% but had no effect on free cholesterol levels. On comparison with the untreated progression control, liver total cholesterol content was significantly reduced 37–94% in all treatment groups except CI-976 (5 mg/kg) and niacin. Liver free cholesterol content was decreased in all treatment groups. The cholesteryl ester to total cholesterol ratio was typically 0.68–0.85 in all treatments; however, the cholestyramine/niacin combination reduced the ratio to 0.57.

**Arterial Wall Lipid Content and Composition**

The cholesteryl ester, free cholesterol, and total phospholipid content of the iliac–femoral lesion is tabulated in Table 2. Statistical comparisons relative to time zero and progression control were made. The percent change in the thoracic aortic cholesteryl ester content relative to the untreated progression control is graphically depicted in Figure 3. Since the thoracic aorta was used to assess whether treatment would alter lesion progression, no comparisons were made relative to the time-zero control.

In the iliac–femoral lesion, CI-976 at 25 mg/kg significantly reduced the cholesteryl ester content 32% relative to the progression control (Table 2). A

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**Table 1. Liver Cholesterol Distribution**

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Total cholesterol (mg/liver)</th>
<th>Free cholesterol (mg/liver)</th>
<th>Cholesteryl ester/total cholesterol</th>
</tr>
</thead>
<tbody>
<tr>
<td>Time zero</td>
<td>2,405±269*</td>
<td>525±22*</td>
<td>0.78</td>
</tr>
<tr>
<td>Progression control</td>
<td>3,878±312</td>
<td>963±65</td>
<td>0.75</td>
</tr>
<tr>
<td>Regression control</td>
<td>1,199±154†</td>
<td>340±38†</td>
<td>0.72</td>
</tr>
<tr>
<td>CI-976</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>5 mg/kg</td>
<td>2,918±277</td>
<td>465±48*</td>
<td>0.84</td>
</tr>
<tr>
<td>25 mg/kg</td>
<td>2,427±326*</td>
<td>370±54†</td>
<td>0.85</td>
</tr>
<tr>
<td>Niacin, 200 mg/kg</td>
<td>3,474±364†</td>
<td>468±39*</td>
<td>0.87</td>
</tr>
<tr>
<td>Cholestyramine, 500 mg/kg</td>
<td>689±405†</td>
<td>219±98†</td>
<td>0.68</td>
</tr>
<tr>
<td>Cholestyramine/niacin, 500:200 mg/kg</td>
<td>228±50†</td>
<td>99±14†</td>
<td>0.57</td>
</tr>
</tbody>
</table>

Data are expressed as mean±SEM.

*Significantly different from progression control at *p*<0.05.
†Significantly different from time-zero control at *p*<0.05.

**Table 2. Lipid Composition of Iliac–Femoral Lesions**

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Cholesteryl ester*</th>
<th>Free cholesterol*</th>
<th>Total phospholipids*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Time zero</td>
<td>125±16</td>
<td>44±3</td>
<td>30±2</td>
</tr>
<tr>
<td>Progression control</td>
<td>133±13</td>
<td>47±3</td>
<td>29±2</td>
</tr>
<tr>
<td>Regression control</td>
<td>122±14</td>
<td>42±3</td>
<td>30±2</td>
</tr>
<tr>
<td>CI-976</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>5 mg/kg</td>
<td>123±19</td>
<td>47±7</td>
<td>47±13†</td>
</tr>
<tr>
<td>25 mg/kg</td>
<td>91±16†</td>
<td>38±6</td>
<td>27±2</td>
</tr>
<tr>
<td>Niacin, 200 mg/kg</td>
<td>159±24</td>
<td>54±5</td>
<td>40±3‡</td>
</tr>
<tr>
<td>Cholestyramine, 500 mg/kg</td>
<td>135±30</td>
<td>37±6</td>
<td>29±5</td>
</tr>
<tr>
<td>Cholestyramine/niacin, 500:200 mg/kg</td>
<td>116±18</td>
<td>33±4‡</td>
<td>25±2</td>
</tr>
</tbody>
</table>

*Data are expressed as mean±SEM in micrograms per milligram dry defatted tissue.
†Significantly different from progression control at *p*<0.05.
‡Significantly different from time-zero control at *p*<0.05.
27% reduction in cholesteryl ester content was noted when compared with the time-zero control; however, this decrease was not statistically significant. CI-976 at 5 mg/kg, diet intervention, or the other pharmacological agents did not alter the cholesteryl ester content of the iliac-femoral lesion. No change in free cholesterol content was noted with either dose of CI-976. The cholestyramine/niacin combination, however, did lower the free cholesterol content 25% relative to the time-zero group. Phospholipid content was elevated 63% with CI-976 (5 mg/kg) and 33% with niacin alone on comparison with the progression control, while no changes were observed with the other treatment regimens.

When the average percent lipid composition was calculated for the iliac-femoral lesions, the time-zero control and progression control contained 63% cholesteryl esters, 22% free cholesterol, and 15% phospholipids. With CI-976 at either dose, the average percent lipid composition was 57–58% cholesteryl ester, 22–24% free cholesterol, and 18–21% phospholipids. No difference from the time-zero or progression control was noted with either dietary intervention, cholestyramine, niacin, or the combination.

CI-976 at both 5 and 25 mg/kg prevented the increase in cholesteryl esters in the thoracic aorta by 46% and 51%, respectively (Figure 3). The cholesteryl ester content after dietary intervention was reduced 44%. Cholestyramine, niacin, or the combination prevented the accumulation of cholesteryl esters by 50%, 20%, and 61%, respectively. The average percent lipid composition of the thoracic aorta was 59% cholesteryl esters, 23% free cholesterol, and 18% phospholipids, and no differences among the groups were observed. No changes in free cholesterol or phospholipid content were observed, and the cholesteryl ester to total cholesterol ratio ranged from 0.65 to 0.76 for all treatments.

Morphological Appearance of the Iliac–Femoral Lesion

A definite, raised lesion was present in 99% of the animals at the aortic–iliac bifurcation at the initiation of diet or pharmacological intervention. The lesion was present on the medial wall of the iliac artery and extended into the femoral artery to the point where the nylon monofilament was secured. Multiple branch vessels were observed between the site of total ligation and the aortic bifurcation.

The general histological appearance of the iliac–femoral lesion before and after intervention was similar; however, the size and extent of lesion components, that is, macrophages and lipid, appeared to differ (Figure 4). The iliac–femoral lesions were characterized by both extensive SMC proliferation and extracellular matrix deposition. SMCs were scattered throughout the lesion. The SMCs were arranged perpendicular to the internal elastic lamina in small lesions, while in larger lesions, the SMCs were organized into layers and formed a fibrous cap. Collagen identified as eosinophilic bands was noted between the SMC layers and amidst monocyte–macrophage foam cell regions. No signs of lesion complications such as cholesterol crystals, calcium deposits, or intimal–medial necrosis were evident. The integrity of the endothelial lining was not apparent in cross section at the level of resolution of the light microscope. On occasion, thrombi were noted within or overlying the lesion; however, no increased probability of thrombi could be attributed to any specific treatment group. When present, the thrombi contained brightly eosinophilic lamellar structures associated with small basophilic particles identified as platelets.

The monocyte–macrophage was a major component of the iliac–femoral lesion. The lipid-filled monocyte–macrophage was identified on the basis of several histological appearances (Figure 5). In hematoxylin- and eosin-stained sections, the monocyte–macrophage appeared as an enlarged, rounded cell with a central nucleus and an abundance of punctate, basophilic granules in the cytoplasm (Figure 5a). In contiguous sections stained for their esterase activity, the monocyte–macrophage was identifiable as a deep purple–black deposit with a very granular appearance (Figure 5b). These esterase-positive cells, when viewed under polarized light, were brightly birefringent and appeared to be an aggregate of lipid droplets with the classically described formée cross appearance (Figure 5c). When the sections were stained specifically for lipid with oil red O, these highly basophilic, esterase-positive, birefringent cells appeared as areas of globular staining with well-defined boundaries (Figure 5d). The globular cell-specific staining was contrasted by the more finely granular extracellular lipid staining.
With diet or pharmacological intervention, the appearance of the monocytes–macrophages did not change, but the apparent area of such cells was altered. As is evident in Figure 6, the relative extent of esterase-positive cells within the lesions varied. Lesions from the progression control, time-zero control, regression control, and niacin treatment groups possessed similar extents of esterase-positive cells (Figures 6a and 6b), while in lesions from animals treated with either CI-976 at both 5 and 25 mg/kg, cholestyramine, or the cholestyramine/niacin combination, the area of macrophages was decreased relative to the other treatment groups (Figures 6c and 6d). It was unclear whether the apparent differences were due to a decrease in macrophage size or number.

In adjacent sections stained for lipids, there was an apparent difference in the degree of intracellular and extracellular lipid staining after CI-976 treatment. After CI-976 treatment, most of the lipid staining appeared as finely granular oil red O droplets with scant globular staining (Figure 7c). In the other treatment groups, no apparent difference in staining pattern was noted (Figures 7a, 7b, and 7d).

Morphometric Analyses

The area of monocyte–macrophage foam cells and the total iliac–femoral lesion area were determined and are graphically depicted in Figure 8. In addition, the percentage of the descending thoracic aorta covered by atherosclerotic lesions and their gross appearance have also been assessed (Table 3, Figure 9).

It is apparent from Figure 8 that in the iliac–femoral artery at the initiation of diet or drug intervention, the average lesion size was 0.506 mm². Monocytes–macrophages accounted for 51% of the total lesion area. Without intervention, the iliac–femoral lesion doubled in size and the area of macrophages increased by 92% over the final 8 weeks of the study. In animals that were switched to normal chow, the lesions continued to progress, and the size and extent of foam cell involvement were similar to the untreated progression control. The size of the lesions after CI-976 treatment was similar to the lesions observed at initiation of drug treatment; however, relative to the progression control, the lesion area was significantly reduced 38–46% at both...
FIGURE 5. Photomicrographs showing appearance and method of identification of monocytes-macrophages within the iliac-femoral lesion. Panel a: After hematoxylin and eosin staining, monocytes-macrophages (asterisk) are characterized as vacuolized cells with punctate granular cytoplasmic staining surrounding a rounded nucleus. Hematoxylin and eosin stain, ×325. Bar=25 μm. Panel b: Esterase-positive stained sections for monocyte-macrophage identification. A dark black precipitate was present in areas of macrophages (asterisk). α-Naphthyl esterase stain, ×325. Bar=25 μm. Panel c: The area of monocytes-macrophages (asterisk) also colocalized with brightly birefringent lipid droplets. Unstained section viewed under polarized light, ×325. Bar=25 μm. Panel d: Section contiguous to that noted in Figure 5c depicts two types of lipid staining within the area of monocytes-macrophages (asterisk). Globular staining pattern indicative of intracellular lipid and the widely dispersed, more granular staining pattern representative of extracellular lipid are apparent. Oil red O stain, ×325. Bar=25 μm.

Doses of the ACAT inhibitor. The macrophage foam cell area was also significantly decreased 62–63% relative to the progression control. When compared with the time-zero control, the lesion size after CI-976 treatment was comparable, but the foam cell area was reduced by 27–29%. This decrease in foam cell area, however, was not statistically different from the time-zero control. After cholestyramine or cholestyramine/niacin treatment, the iliac-femoral lesion size and macrophage foam cell area were significantly reduced, by 40–45% and 67–69%, respectively, relative to the progression control. Niacin treatment alone increased the lesion size and macrophage area by 174% and 110%, respectively, relative to the time-zero control. The ratio of macrophage foam cell area to total lesion area was also reduced with CI-976, cholestyramine, and cholestyramine/niacin, from 0.51 in the time-zero control to...
FIGURE 6. Photomicrographs showing distribution and abundance of esterase-positive monocytes–macrophages within the same iliac–femoral lesions noted in Figure 4. Photomicrographs are representative of lesions exhibiting the mean macrophage area observed with each treatment and are depicted graphically in Figure 8. Panel a: Progression control. Panel b: Regression control or niacin therapy. Panel c: CI-976 when dosed at 5 and 25 mg/kg. Esterase-positive cells are scattered throughout the eccentric atherosclerotic lesion. Panel d: Cholestyramine or cholestyramine/niacin combination. α-Naphthyl esterase stain, ×25. Bar=400 μm.

0.28–0.34. Thus, the changes observed at the level of the arterial wall are similar for CI-976, cholestyramine, and the cholestyramine/niacin combination. It is also noteworthy that no differences in medial area or total vessel area were noted among any of the treatment groups.

With respect to the thoracic aorta, definite raised lesions with a yellowish to pearly white fibrous appearance were observed covering the vessel (Figure 9). It is apparent from Figure 9 that the lesions were typically located about the intercostal ostia, but in the more severely involved vessels, the lesions were observed extending longitudinally and ventrally. Before intervention, 39% of the thoracic aorta was covered by atherosclerotic lesions, whereas at the end of the study, 72% of the aorta from the untreated control contained lesions (Table 3). After CI-976 treatment with either 5 or 25 mg/kg, only 44% or 31% of the thoracic aorta, respectively, was covered by atherosclerotic lesions. Forty-four percent of the thoracic aorta contained lesions after dietary intervention, while only 37% was observed with either cholestyramine or cholestyramine/niacin treatment. Niacin treatment alone was less effective (50%). All treatments were statistically different from the progression control, but they were not different from the time-zero group (Table 3).

Discussion

Inhibition of ACAT within the arterial wall by the potent and specific ACAT inhibitor CI-976, even in the absence of plasma cholesterol lowering, can result in the inhibition of atherosclerotic lesion progression and can enhance regression. This conclusion is supported by several findings of the present study, which can be outlined as follows: 1) Dietary intervention alone increased the monocyte–macrophage involvement in the iliac–femoral lesion despite reductions in plasma, liver, and thoracic aortic cholesterol ester content. 2) CI-976 administered in a hypercholesterolemic diet and at a dose that did not lower plasma cholesterol (5 mg/kg) prevented the accumulation of monocytes–macrophages within the preestablished iliac–femoral lesion and reduced the
FIGURE 7. Photomicrographs showing distribution and characteristics of lipid within iliac–femoral lesions (sections contiguous to those in Figures 4 and 6). Panel a: Progression control. Abundant lipid staining is present throughout the lesion and in portions of the media underlying the lesion. Panel b: Regression control or niacin treatment. Lipid appearance and distribution are similar to those of progression control noted in Figure 7a. Panel c: CI-976 at both 5 and 25 mg/kg. Note the striking paucity of lipid staining within the lesion and the more granular appearance of the staining compared with that in Figure 7b. Panel d: Cholestyramine or cholestyramine/niacin. Oil red O stain, ×25. Bar=400 μm.

foam cell area by 27–29% relative to the initiation of pharmacological intervention. At this same dose, CI-976 also prevented the development of thoracic aortic fatty streak-like lesions and decreased the cholesteryl ester enrichment by 46%. 3) CI-976 at either 5 or 25 mg/kg had no effect on plasma triglycerides and, more importantly, had no effect or decreased the free cholesterol content in the liver, iliac–femoral artery, and thoracic aorta. 4) Conventional lipid-lowering therapy such as cholestyramine or a cholestyramine/niacin combination required substantial decreases in plasma cholesterol levels,

FIGURE 8. Bar graph of morphometric evaluation of the iliac–femoral lesion. Data are expressed as the mean esterase-positive monocyte–macrophage area and total lesion area. Asterisk associated with specific treatments denotes that the monocyte–macrophage foam cell area and total lesion area are significantly different from progression control at p<0.10. Progr, progression control; Regr, regression control; CME, cholestyramine.
TABLE 3. Percent Lesion Coverage of the Descending Thoracic Aorta

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Percent lesion coverage</th>
</tr>
</thead>
<tbody>
<tr>
<td>Time zero</td>
<td>39±12*</td>
</tr>
<tr>
<td>Progression control</td>
<td>72±6</td>
</tr>
<tr>
<td>Regression control</td>
<td>43±7*</td>
</tr>
<tr>
<td>CI-976</td>
<td></td>
</tr>
<tr>
<td>5 mg/kg</td>
<td>44±8*</td>
</tr>
<tr>
<td>25 mg/kg</td>
<td>31±7*</td>
</tr>
<tr>
<td>Niacin, 200 mg/kg</td>
<td>50±8*</td>
</tr>
<tr>
<td>Cholestyramine, 500 mg/kg</td>
<td>37±14*</td>
</tr>
<tr>
<td>Cholestyramine/niacin, 500:200 mg/kg</td>
<td>37±7*</td>
</tr>
</tbody>
</table>

*Significantly different from progression control at p<0.05.

that is, 74–96%, to achieve the same reduction in macrophage foam cell area, thoracic aortic lesion coverage, and cholesteryl ester content. In addition to establishing the role of ACAT in atherosclerotic lesion formation, the present study is unique in that both regression of a preestablished iliac-femoral lesion and progression of thoracic aortic lesions can be evaluated within the same animal model.

Plasma cholesterol levels were decreased 44–97% by both diet and pharmacological intervention; however, the 5 mg/kg dose of CI-976 did not significantly decrease the preestablished hypercholesterolemia. The reductions in plasma cholesterol are consistent with previously published reports describing the activity of specific ACAT inhibitors. CL277082 and cyclandelate at doses of 0.25% and 0.5%, respectively, in the diet (approximately 125 and 250 mg/kg, respectively) decreased a preestablished hypercholesterolemia by 67–93%,13,14 Melinamide28 and a furobenzochromone described by Gammill and colleagues29 were reported to blunt a diet-induced hypercholesterolemia by 46% and 88%, respectively. Kelley et al30 have reported that after 6 weeks of treatment with CL277082, plasma triglycerides were elevated eightfold. In contrast to CL277082, plasma triglycerides were unaffected by CI-976. However, the ACAT inhibitor melinamide has been shown to lower plasma triglycerides at doses greater than 100 mg/kg in rabbits.28 Thus, one might hypothesize that the elevation in plasma triglycerides observed with CL277082 may not be related to the inhibition of ACAT but rather to other unknown actions of the compound.

Hypercholesterolemia in the presence and absence of chronic endothelial injury has been shown to induce atherosclerotic lesions within the iliac–femoral artery and the thoracic aorta.17,18 Before diet or

![Figure 9](https://example.com/figure9.png)
pharmacological intervention and in the untreated progression control, a fibrofatty, lipid-filled, macrophage- and smooth muscle cell–enriched lesion was present within the injured iliac–femoral artery.18 The histological appearance of the iliac–femoral lesion was consistent with that of a human fatty streak lesion; however, the distribution of the cellular elements differed. Namely, lipid-filled, esterase-positive monocytes–macrophages were seen scattered throughout the lesion rather than localized to the superficial intimal layers. Consistent with the morphological evidence, the iliac–femoral lesions and the naturally occurring thoracic aortic lesions were cholesteryl-ester enriched. Fifty-nine percent to 63% of the total lipid was cholesteryl ester, 22–24% was free cholesterol, and 15–18% was phospholipids. The lipid composition of both vascular lesions is similar to those previously reported for human aortic intermediate lesions and fibrolipid lesions.31,32 Thus, the atherosclerotic lesions are comparable to human fatty streak lesions.

Reduction in plasma cholesterol by dietary intervention alone was sufficient to prevent the progression of the naturally occurring thoracic aortic lesions. However, in the more complicated iliac–femoral lesions of the diet intervention control, no difference was observed in the cholesteryl ester content or extent of monocyte–macrophage involvement. Such data is consistent with previously published reports that suggest that preestablished rabbit atherosclerosis cannot be altered by dietary intervention.19 CI-976 when given at 25 mg/kg in the cholesterol plus fat diet decreased iliac–femoral cholesteryl ester content 32% relative to the untreated control. CI-976 at 5 mg/kg, cholestyramine, niacin, or the cholestyramine/niacin combination had no effect on the cholesteryl ester content of the lesion. On histological evaluation, CI-976 at both doses decreased the area of lipid-filled, esterase-positive monocytes–macrophages within these same lesions. The apparent extent of globular oil red O staining was also reduced despite the absence of marked changes in lesion total cholesteryl ester content. The absence of changes in ester content at the 5 mg/kg dose of CI-976 may be indicative of a decreased intracellular pool identified as globular oil red O staining and an increased extracellular pool of lipid. Due to the brevity of the present study, that is, 8 weeks, the extracellular lipid pool may represent the initial stages of reverse cholesterol transport and lesion regression.

An alternative explanation for the changes in macrophage area may be related to the number and size of these cells within the lesion. For instance, in the progression and regression control groups, there was a doubling in the area of monocytes–macrophages within the iliac–femoral lesion relative to the time-zero control; however, there was no increase in lesion cholesteryl ester content. These data suggest that the increased macrophage area may be the result of an increased number of cells containing the same amount of lipid. In contrast, CI-976 at 25 mg/kg decreased both the area of macrophages and the cholesteryl ester content of the lesion. One might speculate that the reduction in macrophage area may be the result of a decreased number of cells; however, it is entirely possible that the macrophages are smaller and less cholesteryl-ester enriched. Further studies are clearly warranted to assess the effect of CI-976 on monocyte–macrophage biology.

In thoracic aortic lesions, cholesteryl ester content and the percentage of the descending thoracic aorta covered by lesions after CI-976 treatment were similar to those of the dietary intervention group. It is noteworthy that the blunted progression and/or regression of the iliac–femoral lesions and thoracic aortic lesions occurred not only at the 25 mg/kg dose of CI-976 but also at the 5 mg/kg dose, which had no effect on plasma and liver cholesterol levels. The observation that antiatherosclerotic activity can be achieved in the absence of plasma cholesterol lowering is in contrast to previously published reports with other ACAT inhibitors. Schaffer and coworkers13 reported that administration of CL277082 to rabbits for 16 weeks after a 10-week lesion induction phase resulted in a 49% reduction in aortic cholesteryl ester content. However, in this same study CL277082 also reduced plasma cholesterol approximately 67%, to 200 mg/dl. Cyclandelate, when given to rabbits in a low-cholesterol chow diet after a similar lesion induction phase, was shown to blunt the increase in aortic total cholesterol content.14 Unlike that in the present study, the cholesterol content of the aorta increased 121–165% relative to pretreatment with dietary intervention and 52–72% with cyclandelate treatment. Plasma cholesterol levels were again markedly reduced. In addition, melinamide28 and a furobenzochromone reported by Gammill et al29 were shown to prevent lesion formation in the cholesterol-fed rabbit; however, the interpretation of the data is again complicated by the decreases in plasma cholesterol. Thus, in contrast to previous studies, changes in lesion morphology and lipid composition after administration of 5 mg/kg CI-976 for 8 weeks appear to be directly related to ACAT inhibition rather than to plasma cholesterol lowering.

The reduction in lipid-filled monocyte–macrophage involvement in the iliac–femoral lesions suggests that CI-976 is present within the extracellular milieu of the lesion and is potentially capable of inhibiting macrophage ACAT. We have observed that after a single oral dose of 25 mg/kg CI-976 to hypercholesterolemic rabbits, a peak plasma level of 0.304 µg/ml (0.77 µM) was achieved (A. Black, personal communication). We have previously found that the median inhibiting concentration for ACAT inhibition in isolated mouse peritoneal macrophages was 0.61 µM (0.24 µg/ml).15 Direct quantification of ACAT inhibition within the vessel was not measured due to the questionable interpretability of the results. For instance, an absence of inhibition within the vessel by CI-976 may be the result of washing of the
drug from the microsomal preparation during isolation rather than an absence of tissue drug levels. Thus, one might conclude from the plasma drug levels that sufficient levels of CI-976 were present in the plasma to inhibit macrophage ACAT, and from the results obtained with the 5 mg/kg dose, one might suspect that interstitial levels of CI-976 were also high enough to affect arterial wall ACAT.

Similar changes in monocyte–macrophage involvement in the iliac–femoral lesions and in thoracic aortic cholesteryl ester content and lesion distribution were observed after cholestyramine and cholestyramine/niacin treatment. Unlike those after CI-976 treatment, plasma cholesterol levels were reduced 74% by cholestyramine and 96% by cholestyramine/niacin. Another obvious difference between the resin or resin/niacin treatment was that the dose of resin was 20 to 100 times that of CI-976 and for niacin, the dose was eight to 40 times greater. It is tempting to draw the analogy with the Cholesterol-Lowering Atherosclerosis Study (CLAS), in which colestipol and colestipol/niacin were evaluated in human subjects and the effect of therapy was measured angiographically. The doses administered to rabbits on a milligram per kilogram of body weight basis were similar to those used in the CLAS study. We have observed that the resin/niacin combination blunted the development of thoracic aortic lesions. In human volunteers, a resin/niacin combination was also found to significantly decrease the percentage of subjects with new coronary lesions. Thus, one might conclude that to obtain antiatherosclerotic effects at the arterial wall with conventional lipid-lowering therapy, profound plasma cholesterol lowering must be achieved, or the lipid-lowering drug must have other pharmacological properties necessary for regression of atherosclerotic lesions.

Therefore, the major implication of the present study is that inhibition of ACAT by CI-976 at the level of the arterial wall may decrease the involvement of monocytes–macrophages within the lesion, blunt lesion progression, and potentiate lesion regression. In addition, conventional lipid-lowering therapy with cholestyramine or a cholestyramine/niacin combination is effective at blunting lesion progression; however, marked plasma cholesterol lowering appears to be necessary.

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