Modulating Effects of Canine High Density Lipoproteins on Cholesteryl Ester Synthesis Induced by β-Very Low Density Lipoproteins in Macrophages

Possible In Vitro Correlates with Atherosclerosis

Thomas L. Innerarity, Robert E. Pitas, and Robert W. Mahley

We have previously observed that cholesterol-fed dogs with plasma cholesterol levels of 350 to 750 mg/dl failed to develop atherosclerosis (hyporesponders), whereas cholesterol-fed dogs with cholesterol levels greater than 750 mg/dl developed markedly accelerated atherosclerosis (hyperresponders). Two striking features of the hypercholesterolemia of the hyperresponders were the occurrence of cholesteryl ester-rich, β-migrating very low density lipoproteins (β-VLDL) in the d < 1.006 fraction and a decrease in plasma concentration of typical high density lipoproteins (HDL). Cholesterol-induced β-VLDL have been shown to cause massive accumulations of cholesteryl esters in mouse peritoneal macrophages in vitro, and HDL have been shown to remove cholesterol from these cells.

In the present study, the mouse peritoneal macrophage system was used to explore the effects of high levels of cholesterol-induced d < 1.006 lipoproteins and low levels of HDL in mediating cholesteryl ester synthesis and accumulation in these cells. It was found that the d < 1.006 lipoproteins from both the atherosclerotic hyperresponders and the nonatherosclerotic hyporesponders stimulated cholesteryl [14C]-oleate synthesis. The d < 1.006 lipoproteins from control chow-fed dogs were not capable of inducing cholesteryl ester formation. The ability of the d < 1.006 lipoproteins from the hypercholesterolemic dogs to stimulate cholesteryl ester synthesis in macrophages was best predicted by the enrichment of these lipoproteins with cholesterol and cholesteryl esters. The modulating effect of HDL was shown by the marked decrease in cholesteryl ester formation and accumulation when macrophages were incubated with both HDL and hypercholesterolemic d < 1.006 lipoproteins. The ratio of HDL cholesterol to d < 1.006 lipoprotein cholesterol added to the macrophages was predictive of the degree of cholesteryl ester formation and accumulation that occurred. HDL with apoprotein E (apo E) and HDL without apo E were capable of reversing the cholesteryl ester formation induced by hypercholesterolemic d < 1.006 lipoproteins; however, the HDL without apo E were more effective. When the ratio of HDL cholesterol to d < 1.006 lipoprotein cholesterol added to the macrophages was > 0.5 to 1.0, there was a marked reduction in the ability of the hypercholesterolemic d < 1.006 lipoproteins to induce significant cholesteryl ester formation. When the ratios of HDL cholesterol to d < 1.006 cholesterol were less than 0.5, the amount of [14C]oleate incorporated into the cholesteryl esters of the macrophages was progressively increased. It is reasonable to speculate that an important determinant of whether cholesteryl esters accumulate in the macrophage model system, and possibly in the arterial wall, is the ratio of those lipoproteins capable of delivering cholesterol to the cells (e.g., β-VLDL, cholesterol-enriched d < 1.006 lipoproteins) to those lipoproteins capable of removing cholesterol from the cells (e.g., HDL). More direct parallels between the in vivo and in vitro observations remain to be determined.

(Arteriosclerosis 2: 114–124, March/April 1982)
CHOLESTEROL ACCUMULATION IN MACROPHAGES Innerarity et al.

derived from the uptake of cholesteryl ester-rich plasma lipoproteins. The uptake of lipoproteins by peritoneal macrophages has been intensively studied in vitro. Massive accumulations of cholesteryl esters result when macrophages are incubated with two specific types of abnormal lipoproteins, whereas normal plasma lipoproteins are taken up poorly by these cells. The two classes of lipoproteins that lead to the massive accumulation of cholesteryl esters in macrophages are internalized through two separate receptor sites. First, it has been shown that low density lipoproteins (LDL) chemically modified by acetylation, 10 cephalonan, or malondialdehyde 11 are taken up by the same receptor site on macrophages. Second, β-migrating very low density lipoproteins (β-VLDL), which are lipoproteins induced by cholesterol feeding, are taken up by macrophages through a separate receptor site. The β-VLDL are cholesteryl ester-rich lipoproteins that float in the d < 1.006 ultracentrifugal fraction and have β-electrophoretic mobility. 12-14 The β-VLDL are the only naturally occurring lipoproteins capable of causing cholesteryl ester accumulation in macrophages. Incubation of macrophages in vitro with either chemically modified LDL or diet-induced β-VLDL converts these cells to cells that have the histologic characteristics of aortic foam cells. The cholesteryl ester droplets that accumulate in the cytoplasm in response to β-VLDL are not metabolically inert. The stored cholesteryl ester is part of a metabolic cycle and is continually undergoing hydrolysis by neutral cholesteryl esterase. The hydrolyzed free cholesterol is re-esterified by the microsomal acyl-CoA:cholesterol acyltransferase (ACAT), thus completing the cycle. The presence of HDL in the medium interrupts this cycle by promoting the net hydrolysis and excretion of the stored cholesteryl esters. These results suggest that the amount of intracellular cholesteryl ester that will accumulate in cultured macrophages depends on one or both of two factors: 1) the concentration of β-VLDL capable of delivering cholesterol to the cells, and 2) the level of HDL in the medium capable of promoting cholesteryl ester efflux from the cells. An understanding of these two processes could contribute to an understanding of the mechanisms involved in both the development and regression of diet-induced atherosclerotic lesions. Previously, we had observed that thyroidectomized dogs fed diets rich in cholesterol and lard could be grouped into two categories based on their response to the diet. Similar results also occurred in dogs fed a coconut oil-cholesterol diet. One group of animals, designated hyperresponders, had plasma cholesterol levels greater than 750 mg/dl and developed accelerated atherosclerosis within a few months. The other group of animals, the hyporesponders, had plasma cholesterol levels below 750 mg/dl and their arteries were devoid of atherosclerotic lesions. Although the hyporesponders had lower plasma cholesterol levels than the hyperre-
exposure to iodine vapors. The adsorbent containing the [7(n)3H]cholesteryl oleate was scraped and the product eluted from the adsorbent with two 5-ml aliquots of benzene. The radiochemical purity of the product was determined by thin-layer chromatography to be greater than 99%.

**Lipoprotein Isolation**

Canine lipoproteins were isolated from the plasma of dogs fed a commercial dog chow (Ralston Purina), or dogs fed a semisynthetic diet containing hydrogenated coconut oil and cholesterol (Teklad Mills, Madison, Wisconsin). The d < 1.006 lipoproteins were isolated from plasma by ultracentrifugation in a 60 Ti rotor (Beckman) at 50,000 rpm for 18 hours at 4°C and washed by recentrifugation at the same density and speed for 16 hours. The canine HDL (d = 1.063 to 1.21) were isolated in a 60 Ti rotor by ultracentrifugation at 59,000 rpm for 48 hours and washed by recentrifugation at 59,000 rpm for 24 hours. In one experiment, the total spectrum of canine lipoproteins was isolated from 4 ml of plasma by the density gradient procedure of Redgrave et al., except that the samples were centrifuged for 48 hours instead of 24 hours. The canine HDL were fractionated by heparin-Sepharose chromatography into HDL that contained the apoprotein E (apo E) and HDL without apo E by the procedure of Weisgraber and Mahley. Lipoproteins were characterized by paper electrophoresis and the apoprotein pattern determined on 11% polyacrylamide disk gels using sodium dodecyl sulfate as described. The lipoproteins were iodinated and classified by preparative thin-layer chromatographic plates and developed in hexane:diethyl ether:ammonium hydroxide (90:10:1; v/v). The cholesteryl ester band was calculated by multiplying the phosphorus content by a factor of 25. The lipoproteins were iodinated by the McFarlane procedure, as modified by Bilheimer et al.

**Cultured Mouse Macrophages**

Mouse peritoneal macrophages were harvested from unstimulated mice using phosphate buffered saline (PBS) as described. The peritoneal lavage from the mice (1 to 3 x 10⁶ cells per mouse) were pooled, and the cells were pelleted by centrifugation (400 x g, 10 min, room temperature). The cells were resuspended at a final concentration of 1.5 x 10⁶ cells per ml in DMEM containing penicillin (100 Units/ml), streptomycin (100 μg/ml), and 20% heat-inactivated fetal calf serum. Either 5.0 x 10⁶, 1.5 x 10⁸, or 3 x 10⁶ cells were dispensed into 16 mm, 35 mm, or 60 mm plastic tissue culture dishes, respectively. After incubation in a humidified CO₂ (7.5%) incubator for 2 hours, the dishes were washed three times with DMEM without serum to remove nonadherent cells. The cultured macrophages were incubated for 24 hours at 37°C in DMEM containing 20% fetal calf serum (0.5 ml for 16 mm dishes, 2 ml for 35 mm dishes, or 3 ml for 60 mm dishes). Then the cells were washed once with DMEM and used in the experiments.

**Cholesteryl [14C]oleate Biosynthesis**

The amount of cholesteryl [1-14C]oleate formed during the incubation of cultured macrophages with [1-14C]oleate complexed to albumin was determined by a procedure similar to a previously described method. At the end of each experiment (see figure or table captions for details of each experiment), the petri dishes of macrophages were chilled on ice, then washed three times in rapid succession with cold PBS, followed by two 10-minute washes, each with PBS containing BSA (2 mg/ml), and then one short wash with cold PBS. The washed macrophages were extracted in situ with hexane:isopropanol (3:2; v/v) for 30 minutes at room temperature. The [3H]cholesteryl oleate was added to the initial extraction solvent to act as an internal standard. The macrophage monolayers were extracted (no added internal standard) two additional times with the same organic solvent, and all three of the extracts were combined in glass conical tubes. After the lipids had been extracted, the macrophages were dissolved with 0.1 N NaOH and aliquots were removed for protein determinations. The lipid extracts were blown dry with a stream of nitrogen and were redissolved with chloroform:methanol (2:1; v/v). The samples were spotted on Whatman LK696 channeled predesorbed thin-layer chromatographic plates and developed in hexane:diethyl ether:ammonium hydroxide (90:10:1; v/v). The cholesteryl ester band was visualized with iodine vapor and scraped into scintillation vials. Hexane:isopropanol (3:2; 0.5 ml) was added to extract the lipids from the silica gel, followed by the addition of 10 ml of Beckman NA L.S. cocktail. The vials were counted using a dual-label ³H,¹⁴C counting program for the Beckman LS-9000.

**Cellular Cholesterol and Cholesteryl Ester Determination**

To determine the mass of cholesterol and cholesteryl ester, the same lipid extraction procedure was used, except the initial extraction solvent contained 5 μg of stigmasterol and 2.0 μg of stigmasteryl oleate to serve as internal standards instead of [3H]cholesteryl oleate. The stigmasteryl oleate was synthesized essentially as described by Patel et al., for the synthesis of cholesteryl esters. The extraction solvent was evaporated and the cholesterol and cholesteryl esters isolated by preparative thin-layer chromatography on 0.5 μm-thick silica gel H plates. The plates were developed in hexane:diethyl ether:acetic acid (90:20:2; v/v). The scraped spots of cholesterol and cholesteryl ester contained the stigmasterol and
stigmasteryl oleate internal standards, respectively. The cholesteryl esters were saponified using methanolic KOH and extracted. The hydrolyzed free cholesterol was extracted with chloroform-methanol (2:1; v/v), and the samples were analyzed by gas liquid chromatography as previously described.24

Results

Previously, it had been shown that cholesteryl ester synthesis in macrophages, as determined by $[^{14}C]$oleate incorporation, correlates directly with cholesteryl ester accumulation in these cells.5,15 In the present studies, cholesteryl ester synthesis was used as a measure of the ability of $d < 1.006$ lipoproteins to deliver cholesterol to macrophages. As shown in table 1, the triglyceride-rich, pre-$\beta$-migrating $d < 1.006$ lipoproteins from control, chow-fed dogs did not stimulate cholesteryl ester synthesis. This confirms previous observations regarding normal dog VLDL.5,6 To explore the possibility that the absence of atherosclerosis in the hypercholesterolemic hyporesponder dogs was due to the inability of their $d < 1.006$ lipoproteins to deliver cholesterol to macrophages, we determined the ability of the hyporesponder $d < 1.006$ lipoproteins to stimulate cholesteryl ester formation. The results shown in table 1 reveal that the hyporesponder $d < 1.006$ lipoproteins markedly stimulated cholesteryl ester synthesis (20-fold increase in $[^{14}C]$oleate incorporation as compared with the values obtained with the control $d < 1.006$ lipoproteins). In fact, it appeared that the $d < 1.006$ lipoproteins from hyporesponders were almost as active in stimulating cholesteryl ester synthesis as were the $d < 1.006$ lipoproteins ($\beta$-VLDL) from the hyperresponders (table 1). The hyporesponder $d < 1.006$ lipoproteins were enriched in cholesterol but retained pre-$\beta$ electrophoretic mobility by paper electrophoresis. The hyperresponder $d < 1.006$ lipoproteins were somewhat more cholesterol-rich (see results below) and migrated with $\beta$-electrophoretic mobility.

The hyporesponder dogs were maintained on the hypercholesterolemic diet for approximately 15 months prior to autopsy and consistently had plasma cholesterol levels of 500 to 600 mg/dl. Despite the hypercholesterolemia and the ability of the $d < 1.006$ lipoproteins to stimulate cholesteryl ester synthesis (and cholesteryl ester deposition) in macrophages, there was a total absence of atherosclerosis in all the major arteries of these animals. As shown in figure 1, the terminal aorta of one of the hyporesponders (2 in table 1) revealed a total absence of lipid staining, as compared with the marked lipid accumulation observed in the aorta of the hyperresponder.

Consideration was given to differences in the plasma lipid levels and to compositional differences among the $d < 1.006$ lipoproteins as possible determinants in predicting the extent of cholesteryl ester synthesis in macrophages. The most predictive parameters were: the ratios of cholesteryl ester/protein ($r = 0.94$), total cholesterol/protein ($r = 0.91$), and cholesterol/triglyceride ($r = 0.78$), in the $d < 1.006$ lipoproteins, and the total plasma cholesterol level of the dogs from which the $d < 1.006$ lipoproteins were obtained ($r = 0.67$) (table 1).

The correlation between the cholesterol/protein ratio of the $d < 1.006$ lipoproteins and the ability of

Table 1. Cholesteryl Ester (CE) Synthesis in Macrophages Induced by Canine Control and Hyperlipidemic $d < 1.006$ Lipoproteins

<table>
<thead>
<tr>
<th>Source of $d &lt; 1.006$</th>
<th>Plasma cholesterol (mg/dl)</th>
<th>Cholesteryl ester synthesis* (nmole/mg)</th>
<th>d &lt; 1.006 lipoprotein characterization†</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control (4)</td>
<td>100–150</td>
<td>&lt; 1.0</td>
<td>—</td>
</tr>
<tr>
<td>Hyporesponder</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>490</td>
<td>27</td>
<td>2.1</td>
</tr>
<tr>
<td>2</td>
<td>600</td>
<td>15</td>
<td>2.1</td>
</tr>
<tr>
<td>Hyperresponder</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>750</td>
<td>39</td>
<td>2.8</td>
</tr>
<tr>
<td>2</td>
<td>800</td>
<td>24</td>
<td>2.3</td>
</tr>
<tr>
<td>3</td>
<td>800</td>
<td>20</td>
<td>2.1</td>
</tr>
<tr>
<td>4</td>
<td>1450</td>
<td>57</td>
<td>4.4</td>
</tr>
<tr>
<td>Correlation with CE synthesis</td>
<td>$r = 0.67$</td>
<td>—</td>
<td>$r = 0.94$</td>
</tr>
</tbody>
</table>

* Determined by measuring $[^{14}C]$oleate incorporation into the cholesteryl esters of peritoneal macrophages incubated with 100 $\mu$g of $d < 1.006$ lipoprotein cholesterol per ml of culture medium. Other conditions are as described in figure 2.

† Ratios as determined from the chemical composition of the $d < 1.006$ lipoproteins.
Figure 1. Photograph of the terminal aortas and iliofemoral arteries of two cholesterol-fed dogs. The aortas are opened, pinned out flat, and the endothelial surface is stained with Sudan IV, as described. Black areas represent the sudanophilic atherosclerotic lesions. A. Foxhound fed the coconut oil-cholesterol diet for ~15 months. The plasma cholesterol levels were consistently in the 500 to 600 mg/dl range (hyporesponder). No detectable atherosclerosis noted in the terminal aorta (shown) or in any major artery examined. B. Aorta from a foxhound fed the coconut oil-cholesterol diet for ~10 months. The plasma cholesterol levels were consistently greater than 750 mg/dl (hyperresponder). Extensive atherosclerosis involved the terminal aorta.

these lipoproteins to stimulate cholesteryl ester synthesis was extended in additional studies using d < 1.006 lipoproteins with cholesterol/protein ratios varying from 2.8:1 to 4.8:1. The hypercholesterolemic d < 1.006 fractions were incubated with macrophages at cholesterol concentrations of 50 and 100 µg/ml, and the incorporation of [14C]oleate into the intracellular cholesteryl esters was measured. An excellent correlation was observed between [14C]oleate incorporation and the cholesterol/protein ratio of the d < 1.006 lipoproteins (figure 2). It is of interest that, when the lines are extrapolated to the ordinate (a point at which no stimulation of cholesteryl ester synthesis occurs), they intersect at a value for the cholesterol/protein ratio of 0.8. This is, in fact, the cholesterol/protein ratio determined for normal dog d < 1.006 lipoproteins that did not stimulate cholesteryl ester synthesis (table 1).

As the plasma cholesterol levels of the cholesterol-fed dogs increased, not only did the composition of the d < 1.006 lipoproteins change, but the concentration of these lipoproteins also increased markedly (table 2). In addition, as previously reported, there were marked reductions in the levels of plasma HDL with cholesterol feeding. These changes can be readily appreciated by comparing the change in the
plasma cholesterol levels in five cholesterol-fed dogs with the change in the ratio of HDL cholesterol/d < 1.006 lipoprotein cholesterol (table 2). From these data, it is also evident that there was a marked increase in the d < 1.006 lipoproteins relative to the HDL (table 2). Consideration was given to the possibility that the ratio of HDL/d < 1.006 cholesterol might be an important determinant of cholesteryl ester synthesis and accumulation in the macrophage model system. Ho et al. have demonstrated that HDL lowers the intracellular content of cholesterol by stimulating the release of cholesterol into the medium. There is an associated reduction in acyl-CoA:cholesterol acyltransferase activity. As shown in figure 3, there was a decrease in cholesteryl ester synthesis induced by hypercholesterolemic d < 1.006 lipoproteins when HDL were included in the incubation medium.

To determine if the effects of HDL were due to the presence of a specific HDL subclass, the HDL were

Table 2. Increase in the d < 1.006 Lipoproteins and Relative Decrease In HDL with Cholesterol Feeding

<table>
<thead>
<tr>
<th></th>
<th>Total plasma cholesterol (mg/dl)</th>
<th>d &lt; 1.006 cholesterol (mg/dl)</th>
<th>Ratio of HDL cholesterol to d &lt; 1.006 cholesterol*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control (10)</td>
<td>100-150</td>
<td>~1.0</td>
<td>70-100</td>
</tr>
<tr>
<td>Cholesterol-fed</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>B</td>
<td>166</td>
<td>1.4</td>
<td>100</td>
</tr>
<tr>
<td>C</td>
<td>225</td>
<td>11.2</td>
<td>9.7</td>
</tr>
<tr>
<td>D</td>
<td>370</td>
<td>34.0</td>
<td>2.5</td>
</tr>
<tr>
<td>E</td>
<td>553</td>
<td>70.7</td>
<td>1.6</td>
</tr>
<tr>
<td>F</td>
<td>659</td>
<td>147.0</td>
<td>0.6</td>
</tr>
</tbody>
</table>

*The ratio of the HDL cholesterol to the d < 1.006 cholesterol was determined by quantitation of HDL levels in the d = 1.063 to 1.21 ultracentrifugal fraction after density gradient centrifugation.
The effect of HDL on cholesteryl ester synthesis induced by the d < 1.006 lipoproteins was modulated by the presence of HDL. As shown in figure 5A, the addition of 100 μg/ml of hypercholesteremic HDL resulted in more than a sixfold increase in cholesteryl ester synthesis (~95 nmoles/mg). The effects of high levels of HDL on cholesteryl ester synthesis are illustrated in figure 5B. An HDL cholesterol/d < 1.006 cholesterol ratio of less than 0.5 resulted in a significantly greater synthesis of cholesteryl esters than that observed when the ratio was greater than 0.5. An HDL cholesterol/d < 1.006 cholesterol ratio of less than 1 is frequently observed in atherosclerosis-susceptible hyperresponders.

The effects of HDL on cholesteryl ester accumulation induced by the d < 1.006 lipoproteins were determined directly by measuring the mass of cholesterol and cholesteryl esters in the macrophages. As shown in figure 6, when an increasing concentration of HDL was added to the incubation medium containing 100 μg/ml of hypercholesterolemic d < 1.006 lipoprotein cholesterol, there was a marked reduction in the cholesteryl ester content of the cells. Macrophages incubated with 100 μg/ml of hypercholesterolemic d < 1.006 lipoprotein cholesterol accumulated large quantities of cholesteryl ester (65 μg sterol/1 mg cell protein) compared with cells incubated alone (<1 μg cholesteryl ester/1 mg cell protein). By contrast, 100 μg/ml of d < 1.006 lipoprotein cholesterol incubated with the cells in the presence of 100 μg of HDL cholesterol per ml resulted in the accumulation of only 5 μg cholesteryl ester/1 mg cell protein (figure 6). Cellular free cholesterol also decreased slightly in the presence of an increased concentration of HDL.

To approach more closely the in vivo situation, the plasma lipoproteins isolated at d < 1.21 by ultracentrifugation were incubated with cultured macrophages, and the overall effect of the presence of all plasma lipoproteins on [14C]oleate incorporation into cholesteryl esters was determined. The plasma of the five cholesterol-fed dogs listed in table 2 was raised to the density of 1.21 g/ml and all the lipoproteins floated by centrifugation for 48 hours at 59,000 rpm in a 60 Ti rotor. After dialysis against NaCl, the total plasma lipoprotein fraction was added to the macroparticles at a cholesterol concentration of 100 μg/ml and incubated for 16 hours. The amount of cholesteryl [14C]oleate formed in response to the total plasma lipoproteins from these cholesterol-fed dogs was correlated with the ratio of the HDL to d < 1.006 cholesterol (figure 7). Even with this more complex mixture of plasma lipoproteins, a similar relationship existed between the synthesis of cholesteryl ester and the ratio of HDL cholesterol to d < 1.006 lipoprotein cholesterol.

To determine the mechanism whereby HDL inhibited cholesteryl ester synthesis was due to inhibition of the binding of the hypercholesterolemic d < 1.006 lipoproteins, competitive binding and degradation studies were performed. As shown in figure 8, unlabeled hypercholesterolemic d < 1.006 lipo-
Figure 5. A. Cholesteryl ester formation in cultured mouse macrophages incubated with various concentrations of canine hypercholesterolemic d < 1.006 lipoproteins and canine HDL. Each 16 mm petri dish of macrophages received 0.25 ml of DMEM containing 0.2 mM [14C]oleate, the indicated concentration of canine hypercholesterolemic d < 1.006 lipoproteins, and either no added canine HDL (●), or canine HDL at the following cholesterol per ml concentrations: 25 μg (●), 50 μg (●), or 100 μg (●). The macrophages were incubated for 16 hours at 37° C. The content of cholesteryl [14C]oleate in cells incubated without added lipoproteins was 0.4 nmole/mg. A and B represent the same data plotted differently. In B, the cholesteryl ester synthesized is plotted as a function of the ratio of the HDL cholesterol to d < 1.006 cholesterol.

Figure 6. Free cholesterol and cholesteryl ester content of mouse peritoneal macrophages incubated with 100 μg/ml of hypercholesterolemic d < 1.006 cholesterol and various concentrations of canine HDL. Each 60 mm petri dish of cells received 2.0 ml of DMEM containing the indicated concentrations of lipoproteins. After incubation for 18 hours at 37° C, the cellular content of free and esterified cholesterol was determined as described in the Methods section. The content of free cholesterol in cells incubated without added lipoproteins was 25.8 ng of sterol per mg of cellular protein.

Figure 7. Cholesteryl ester formation in mouse macrophages incubated with canine lipoproteins of the ultracentrifugal density fraction d < 1.21 (100 μg of cholesterol per ml) was plotted as a function of the ratio of HDL cholesterol to d < 1.006 cholesterol. The experimental conditions are the same as described in figure 2. The ratio of HDL cholesterol to d < 1.006 cholesterol was determined from the lipoproteins isolated by density gradient centrifugation as described in the Methods section.
proteins effectively competed with the $^{125}$I-d $<$ 1.006 lipoproteins and resulted in a decrease in the degradation of these lipoproteins as a function of the increasing concentration of the unlabeled d $<$ 1.006 lipoproteins. However, neither HDL nor the HDL subfractions were capable of significantly decreasing the amount of $^{125}$I-d $<$ 1.006 lipoprotein degradation (figure 8). These results indicate that HDL did not decrease d $<$ 1.006 lipoprotein-induced cholesteryl ester synthesis and accumulation by preventing the binding and internalization of these lipoproteins by the macrophages.

Discussion

The regulation of cholesterol accumulation in macrophages has been used as an in vitro model system with which to explore possible relationships between changes in plasma lipoproteins and the development of atherosclerosis in cholesterol-fed dogs. Previously, it was observed that dogs fed hypercholesterolemic diets developed atherosclerosis when their plasma cholesterol levels exceeded 750 mg/dl (hyperresponders), but not at lower levels which may be as high as 500 to 600 mg/dl (hyporesponders). In the present study, we have used the peritoneal macrophage system to approach at a cellular level the possible role of the changes in the various plasma lipoproteins in the development of diet-induced canine atherosclerosis. Two possible mechanisms were considered to explain the nonatherogenic hypercholesterolemia in the hyporesponders vs the atherogenic hypercholesterolemia of the hyperresponders. One possibility was that the d $<$ 1.006 lipoproteins of the hyporesponders were not capable of delivering cholesterol to cells of the arterial wall; thus, despite very high levels of plasma cholesterol, the appropriate atherogenic lipoproteins were not present. Previously, we had shown that the $\beta$-VLDL of hyperresponders were capable of delivering massive amounts of cholesterol to macrophages, as reflected by an increased cholesteryl ester synthesis. This uptake was mediated by specific macrophage receptors ($\beta$-VLDL receptors), which were distinct from typical LDL receptors and distinct from the receptors capable of interacting with chemically modified LDL. In the present studies, it was shown that the cholesterol-enriched d $<$ 1.006 lipoproteins from the hyporesponders, even though they were not typical, $\beta$-migrating $\beta$-VLDL, were capable of stimulating cholesteryl ester synthesis in macrophages. Presumably, the uptake of these lipoproteins was mediated by the $\beta$-VLDL receptors. In fact, it was shown that, as the plasma cholesterol levels were increased by the high cholesterol diet, there was a progressive increase in the d $<$ 1.006 lipoproteins and a progressive enrichment of these lipoproteins with cholesterol. This increase in cholesterol content of the d $<$ 1.006 lipoproteins was highly predictive of the capability of the lipoproteins to interact with macrophages and stimulate cholesteryl ester synthesis.

The second possibility considered in explaining the apparent susceptibility of hyperresponders to develop atherosclerosis was related to the decrease in HDL levels associated with diet-induced hypercholesterolemia. A reduced level of HDL, combined with increased levels of d $<$ 1.006 lipoproteins capable of delivering cholesterol, might result in a net accumulation of cholesteryl ester. The results from the present in vitro study are consistent with this hypothesis. As the level of HDL was increased in the medium, the hypercholesterolemic d $<$ 1.006 lipoproteins had less and less stimulating effect on the accumulation of cholesteryl $[^{14}$C]oleate and cholesteryl ester mass in the macrophages.
The mechanism whereby HDL modulated the stimulation of cholesteryl ester synthesis induced by the hypercholesterolemic d < 1.006 lipoproteins was only partially defined. It was shown that the HDL were not acting by directly inhibiting the interaction of the d < 1.006 lipoproteins with the macrophages. Furthermore, based on previous studies,15,16 it is reasonable to speculate that HDL stimulated the efflux of cholesterol from the cells. Therefore, because of a deficiency of substrate, and possibly because of a more direct effect of HDL on the inhibition of acyl-CoA:cholesterol acyltransferase, less cholesterol is esterified.15

The present studies represent an attempt to compare in vivo observations with those obtained in simplified model systems in vitro. Interpretation of such studies is difficult. However, it is of interest that several parallels exist. The consistency of the observation that the d < 1.006 lipoproteins of various cholesterol-fed animals are the only naturally occurring plasma lipoproteins capable of causing cholesterol ester accumulation in macrophages is intriguing.5,6 The most active hypercholesterolemic d < 1.006 lipoproteins appear to be of intestinal origin and to be produced in response to a high cholesterol diet (Fainaru, Mahley, Hamilton, and Innerarity, unpublished observation). Furthermore, the indication that the level of HDL serves to modulate the level of cholesterol in the cells is consistent with the postulated role for HDL. The HDL have been shown to promote cholesterol efflux from various cells,25 including macrophages.15 The known association of a reduction in HDL levels in various diet-induced models of experimental atherosclerosis5 is of interest in the light of epidemiologic data indicating that individuals with low levels of HDL cholesterol are at greater risk of developing coronary artery disease.6,7 The interaction of these two changes in the lipoproteins — the presence of d < 1.006 lipoproteins capable of delivering cholesterol to macrophages and the modulating effect of HDL — in determining the overall balance in cholesterol metabolism in macrophages suggests a possible explanation for the in vivo observations. It is reasonable to speculate that once the flux of cholesterol into the cells mediated by the hypercholesterolemic d < 1.006 lipoproteins exceeds the net flux of cholesterol out of cells mediated by HDL, atherosclerosis results. It is suggested that the critical point occurs at a plasma cholesterol level of approximately 750 mg/dl, the level predictive of the presence or absence of atherosclerosis in cholesterol-fed dogs.

Acknowledgments

The authors thank Kay Arnold and Kathy LaSala for excellent technical assistance, Joe F. Andrews and Russell Levine for editorial assistance, and Richard Wolfe for manuscript preparation.

References

17. Patel KM, Sklar LA, Currie HJ, Pownall HJ, Morrisett JD, Sparrow JT. Synthesis of saturated, unsaturated, spin-labeled, and fluorescent esters: Acylation of cholesterol using
fatty acid anhydride and 4-pyrrolidinopyridine. Lipids 1979; 14: 816–818


Index Terms: hyperlipoproteinemia • cholesterol feeding • dietary cholesterol • canine atherosclerosis


Modulating effects of canine high density lipoproteins on cholesteryl ester synthesis induced by beta-very low density lipoproteins in macrophages. Possible in vitro correlates with atherosclerosis.

T L Innerarity, R E Pitas and R W Mahley

doi: 10.1161/01.ATV.2.2.114

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://atvb.ahajournals.org/content/2/2/114.citation

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in Arteriosclerosis, Thrombosis, and Vascular Biology can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial Office. Once the online version of the published article for which permission is being requested is located, click Request Permissions in the middle column of the Web page under Services. Further information about this process is available in the Permissions and Rights Question and Answer document.

Reprints: Information about reprints can be found online at:
http://www.lww.com/reprints

Subscriptions: Information about subscribing to Arteriosclerosis, Thrombosis, and Vascular Biology is online at:
http://atvb.ahajournals.org//subscriptions/