Hepatic Origin of Cholesteryl Oleate in Coronary Artery Atherosclerosis in African Green Monkeys

Enrichment by Dietary Monounsaturated Fat

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Abstract

Relationships among plasma lipoprotein cholesterol, cholesterol secretion by the isolated, perfused liver, and coronary artery atherosclerosis were examined in African green monkeys fed diets containing cholesterol and 35% of calories as fat enriched in polyunsaturated, monounsaturated, or saturated fatty acids. The livers of animals fed monounsaturated fat had significantly higher cholesteryl ester concentrations (8.5 mg/g wet wt) than the livers of the other diet groups (3.65 and 3.37 mg/g wet wt for saturated and polyunsaturated fat groups, respectively) and this concentration was highly correlated with plasma cholesterol and apoB concentrations in each diet group. Cholesteryl oleate was 58 and 74.5% of the liver cholesteryl ester in the saturated and monounsaturated fat groups. In each diet group, perfusate cholesteryl ester accumulation rate was highly correlated to liver and plasma cholesterol concentrations, and to plasma LDL cholesteryl ester content. Cholesteryl oleate was 48 and 67% of the cholesteryl esters that accumulated in perfusate in the saturated and monounsaturated fat animals, and this percentage was very highly correlated ($r = -0.9$) with plasma apoB concentration. Finally, in these two diet groups, liver perfusate cholesteryl ester accumulation rate was well correlated ($r \geq 0.8$) to coronary artery cholesteryl ester concentration, a measure of the extent of coronary artery atherosclerosis that occurred over the five years of diet induction in these animals. These data define an important role for the liver in the cholesteryl oleate enrichment of the plasma lipoproteins in the saturated and monounsaturated fat groups, and demonstrate strong relationships among hepatic cholesteryl ester concentration, cholesteryl ester secretion, and LDL particle cholesteryl ester content. The high correlation between liver cholesteryl ester secretion and coronary artery atherosclerosis provides the first direct demonstration of the high degree of importance of hepatic cholesteryl ester secretion in the development of this disease process. The remarkable degree of enrichment of cholesteryl oleate in plasma cholesteryl esters of the monounsaturated fat group may account for the relatively high amount of coronary artery atherosclerosis in this group. (*J. Clin. Invest.* 1997. 100:74–83.) Key words: polyunsaturated fat • low density lipoproteins • apolipoprotein B • saturated fat • liver perfusion

Introduction

We have recently reported on the influence in African green monkeys of dietary saturated, monounsaturated, and *n*-6 polyunsaturated fatty acids on plasma lipoproteins and coronary artery atherosclerosis (1, 2). Inclusion of saturated fatty acids as the major component of the diet, together with cholesterol, leads to high LDL cholesterol concentrations, enrichment of plasma LDL particles with cholesteryl oleate, and significant amounts of coronary artery atherosclerosis (1). Substitution of monounsaturated fatty acids for some of the saturated fatty acids lowered LDL cholesterol concentrations while keeping HDL high, but promoted an even greater enrichment of LDL particles with cholesteryl oleate at the expense of cholesteryl linoleate. Coronary artery atherosclerosis equivalent in extent to that in the animals fed saturated fat occurred (1). Substitution of polyunsaturated fatty acids (primarily linoleic acid) for saturated fatty acids in the diet resulted in lower LDL cholesterol concentrations and smaller LDL particles with cholesteryl linoleate as the predominant cholesteryl ester. HDL cholesterol concentrations were also lower, but the extent of atherosclerosis was the lowest among the three dietary fat groups (1).

The relatively high amount of coronary artery atherosclerosis in the monounsaturated fat group was surprising when one considers that low LDL and high HDL cholesterol concentrations should be protective against coronary heart disease (CHD)[1] (3–6). One possible reason for this outcome could be that the dietary monounsaturated fatty acid-induced lipoprotein particle enrichment in cholesteryl oleate (7, 8) may be more important than previously suspected, and sufficient to overcome the beneficial antiatherogenic change in LDL to HDL cholesterol ratio. Earlier studies in CHD patients suggested that the percentage of cholesteryl linoleate in plasma lipoproteins was inversely correlated to clinical outcome (9–13). Such findings suggest that the lack of protection by monounsaturated fatty acids against coronary artery atherosclerosis, as described in African green monkeys (1), may indeed be pertinent for humans, i.e., the shift in LDL cholesteryl ester composition may be an important atherogenic factor that should be considered along with LDL and HDL cholesterol levels.

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1. *Abbreviations used in this paper:* ACAT, acyl-Coenzyme A:cholesterol acyltransferase; CHD, coronary heart disease.

In these studies we have sought to identify the metabolic origin of the compositional differences in the plasma lipoproteins and the related coronary artery atherosclerosis of African green monkeys. Our previous data suggest that in nonhuman primates fed atherogenic diets containing saturated fat, LDL particle enlargement is associated with cholesteryl oleate enrichment and is predictive of the extent of coronary artery atherosclerosis (14, 15). The activity of liver microsomal acyl-Coenzyme A:cholesterol acyltransferase (ACAT) has also been shown to be significantly correlated to the extent of coronary artery atherosclerosis (14). The increased abundance in plasma LDL of cholesteryl oleate, the primary product of hepatic ACAT (16), presumably derives from the increase in liver cholesterol content induced by the atherogenic diet and an associated stimulation of hepatic ACAT activity (14).

Using isolated perfused livers from monkeys, we previously found that an atherogenic diet increased secretion of cholesteryl oleate by the liver in apoB-containing lipoproteins (14, 17). When the diet is enriched in oleic acid, plasma lipids become enriched in oleic acid as a result of the increased availability of this fatty acid, as seen in monkeys and humans (7, 18), and tissue lipids also become enriched when an enriched diet is fed for extended periods of time (Rudel, L.L., unpublished observations). We hypothesized that the increased availability of oleic acid in the liver either activates ACAT, an idea suggested much earlier (16), or facilitates increased activity of this enzyme leading to the remarkable enrichment in cholesteryl oleate that has been seen in plasma lipoproteins in monkeys (1, 18) and similarly in man (7, 8). Increased cholesteryl oleate and decreased cholesteryl linoleate in blood plasma are important factors related to an apparent increase in atherogenicity of plasma lipoproteins, as described in humans (8) and monkeys (1).

To provide data for our hypothesis, the livers from the African green monkeys fed monounsaturated fat were isolated and perfused and lipoprotein cholesterol secretion was compared to that in livers of animals fed saturated and polyunsaturated fat. The evidence supports the possibility that overproduction of cholesteryl oleate by the liver causes cholesteryl oleate enrichment of plasma lipoproteins. The data show that cholesteryl ester secretion by the liver, the majority of which is cholesteryl oleate, is strongly and positively correlated to the extent of coronary artery atherosclerosis in these monkeys.

Methods

The monkeys used in these experiments were adult male African green monkeys (*Cercopithecus aethiops*). These groups have been the topic of earlier publications (1, 2). The 44 animals were originally placed into three groups of 14, 15, and 15 animals that were determined to have equivalent plasma total cholesterol and HDL cholesterol responses to an 8-wk challenge with a diet containing saturated fat and cholesterol. Each group was then fed an experimental diet containing 35% of calories as fat and 0.8 mg of cholesterol per kcal. During the experimental period, which lasted 60 mo, each group was fed a diet with a different fatty acid enrichment, including monounsaturated (> 70% oleic acid), polyunsaturated (> 70% linoleic acid), and saturated (> 40% palmitic acid) fatty acids. The fats chosen for the bulk of the experimental period were essentially the same as used in the study of Mattson and Grundy (3). Linoleic acid-rich and oleic acid-rich safflower oil was the source of fat in the polyunsaturated and monounsaturated fat diets, respectively, thus controlling for background constituents of the oils that could affect oxidation, etc. (19). Complete diet compositions were published (2). The group selection, housing, feeding, and monitoring of the animals throughout the study have also been reported (1, 2). Animals were kept under the care of a veterinarian in an American Association for the Accreditation of Laboratory Animal Care approved facility according to a protocol approved by the institutional animal care and use committee.

Plasma lipid, lipoprotein, and apolipoprotein analyses were done according to schedules and procedures described previously (1) on blood samples taken periodically throughout the experiment. The endpoints used for each animal are means of values determined multiple times throughout the experiment, and represent multiple determinations for each animal.

At the end of the experimental period, livers were removed from the animals for recirculating liver perfusion. The perfusion was carried out essentially as described before (14, 20) and the procedure is briefly summarized. After isolation, each liver was freed of trapped plasma lipoproteins by 60 min of recirculating perfusion with glucose, amino acid, insulin, cortisol, and bile acid-enriched Krebs–Henseleit original Ringer bicarbonate buffer containing washed human erythrocytes at a 22% hematocrit. Fresh perfusate replaced that of the flush period, and recirculating perfusion was carried out for 120 min, during which 10-ml aliquots of perfusate were taken every 20 min for assay of lipids and apolipoproteins, so that the accumulation rate during the perfusion period could be estimated (20). At the time of necropsy, the heart was isolated and perfusion fixed with 10% neutral-buffered formalin at 100 mmHg for 1 h. The left anterior descending coronary artery was then dissected free from the heart, cleaned of adventitia, and stored in formalin at 4°C until extraction with chloroform:methanol, 2:1, for measurement of cholesterol and cholesteryl ester (1).

Concentrations of perfusate apolipoproteins, including apoA-I, apoA-II, apoB, and apoE, were assayed using enzyme-linked immunosorbent assays, as described previously (21, 22). Perfusate triacylglycerol, total cholesterol, and unesterified cholesterol were assayed enzymatically (23) and phospholipid phosphorus was assayed after lipid extraction (24). The caudate lobe of the liver was removed after the liver had been flushed of blood but before recirculating perfusion was started, and was snap frozen in liquid nitrogen, and stored at −80°C. At the time of analysis, a portion of the frozen tissue was taken, weighed, and liver lipid concentrations were determined as above after lipid extraction (23). For measurement of cholesteryl ester fatty acid percentage composition, lipids were extracted in chloroform-methanol, 2:1, major classes were separated by thin layer chromatography using Empore TLC sheets (Analytichem International, Harbor City, CA), the cholesteryl ester band was scraped from the plate, and the fatty acids were isolated, methylated, and quantified by gas liquid chromatography (25).

Statistical analyses were performed as indicated. Typically, one-way ANOVA was used to compare for diet effects and the Fisher's preferred least significant difference post hoc analysis was used to differentiate individual diet group effects. Average values±SEM for diet groups are shown throughout. Where indicated, simple correlation and/or linear regression analyses were done. Individual diet groups were first analyzed separately, and then data were combined where relationships permitted.

Results

The dietary fatty acid effects on liver lipid concentrations are shown in Table I. No statistically significant differences were found among dietary fat groups in liver protein or triacylglycerol concentrations. Interestingly, a statistically significant difference ($P < 0.001$) in liver phospholipid concentration was found where the average concentration in the saturated fat group, 21.3±0.8 mg/g wet wt was less than in the polyunsaturated fat (24.4±1.2 mg/g wet wt) and monounsaturated fat (26.9±0.9 mg/g wet wt) groups. Unesterified (free) cholesterol was also significantly different ($P < 0.04$) among groups, and

Table I. Liver Lipid and Protein Concentrations

Diet	*n*	Liver concentration (mg/g wet wt)				
		Protein	Triacylglycerol	Phospholipid	Free cholesterol	Cholesteryl ester
SAT	12	111.90±7.08	6.25±0.39	21.28±0.85	3.11±0.32	3.65±0.68
MONO	13	121.34±5.25	6.99±1.35	26.88±0.92	4.53±0.66	8.46±1.25
POLY	11	106.79±5.34	5.96±0.48	24.40±4.05	2.76±0.21	3.37±0.47
Significance by ANOVA (*P*)		0.23	0.71	0.0012	0.031	0.0005
Fisher's Post Hoc						
Sat vs Mono				0.0003	0.0375	0.0007
Sat vs Poly				0.037	0.63	0.84
Mono vs Poly				0.087	0.0153	0.0006

the concentration in the monounsaturated fat group (4.53±0.7 mg/g wet wt) was higher than in the saturated fat (3.11±0.3 mg/g wet wt) and polyunsaturated fat, (2.76±0.2 mg/g wet wt) groups. The most highly significant difference ($P < 0.0005$) was in liver cholesteryl ester concentration, where the average concentration in the monounsaturated fat group (8.46±1.3 mg/g wet wt) was over twofold higher than for either of the other two groups (3.65±0.7 and 3.37±0.5 mg/g wet wt for the saturated and polyunsaturated fat groups, respectively).

The liver cholesterol response to the atherogenic diet was widely different among individual animals, even within a diet group. Liver total cholesterol concentration was proportional to both cholesteryl ester and free cholesterol concentration and the data for all diet groups fit the same regression lines as shown in Fig. 1. Both relationships were highly significant ($P < 0.001$), as indicated by the correlation coefficients which were $r = 0.91$ for cholesteryl ester and $r = 0.85$ for free cholesterol. The enrichment in cholesteryl ester in animals that responded to a greater extent was more pronounced than the enrichment in free cholesterol. The range of free cholesterol concentrations was $\sim$ 5-fold, from 1.56 to 7.9 mg/g wet wt, while the range for cholesteryl ester was much greater being $\sim$ 30-fold, from 0.65 to 19 mg/g wet wt. The graph illustrates that livers from animals fed monounsaturated fat typically contained the most free cholesterol and cholesteryl ester.

The percentage composition of liver cholesteryl esters was measured and the data are shown in Table II. For each cholesteryl ester except cholesteryl eicosenoate (20:1) and arachidonate (20:4), the percentage was significantly different when compared among diet groups. The cholesteryl ester that predominated when liver cholesteryl ester concentrations were the highest was cholesteryl oleate, averaging 75±2.8% of hepatic cholesteryl esters in the monounsaturated fat group and 58±2.8% in the saturated fat group. Only in the polyunsaturated fat group was cholesteryl oleate at 25±1.9% not the major liver cholesteryl ester. In this group, cholesteryl linoleate was predominant at 40±3.5% of liver cholesteryl ester, presumably reflecting ACAT products when enrichment of the hepatic acylCoA pool with linoleic acid occurs, as when this fatty acid was the predominant dietary fatty acid. The percentage of cholesteryl linoleate in livers of the other groups was significantly less at 9.8±0.8% and 4.8±0.3% in the saturated and monounsaturated fat groups, respectively, values that were between four- and eight-fold lower than in the livers of the polyunsaturated fat group. The highest percentage of cholesteryl palmitate was in livers of the saturated fat group, where it comprised 12.6±0.7% of the cholesteryl esters, and the lowest percentage for this ester was in the livers of the monounsaturated fat group at 3.5±0.5%. The percentage of cholesteryl stearate was also highest in the saturated fat group, 7.9±0.6%, and was lowest in the monounsaturated fat group at 2.5±0.4%. Clearly, the relative proportions of cholesteryl esters in the liver were highly diet dependent.

At the time of necropsy, each liver was removed and perfused in a recirculating system to identify any effects of diet on apolipoprotein and lipid accumulation during perfusion. As was shown in an earlier publication (20), accumulation rates of lipids and apolipoproteins are linear throughout 120 min of

Table II. Liver Cholesteryl Ester Percentage Composition

Diet	*n*	Liver cholesteryl ester fatty acid (% wt/wt)						
		14:0	16:0	18:0	18:1	18:2	20:1	20:4
SAT	12	1.1±0.3	12.6±0.7	7.9±0.6	58.0±2.8	9.8±0.8	2.5±1.2	1.2±0.6
MONO	13	0.4±0.1	3.5±0.5	2.5±0.4	74.5±2.8	4.8±0.3	4.4±0.9	1.9±0.5
POLY	11	1.2±0.3	7.2±1.4	5.6±1.0	25.4±1.9	40.0±3.5	8.0±2.9	1.9±1.0
Significance by ANOVA (*P*)		0.01	0.0001	0.0001	0.0001	0.0001	> 0.1	> 0.1
Fisher's Post Hoc								
Sat vs Mono		0.01	0.0001	0.0001	0.0001	0.07		
Sat vs Poly			0.0003	0.02	0.0001	0.0001		
Mono vs Poly		0.01	0.009	0.002	0.0001	0.0001		

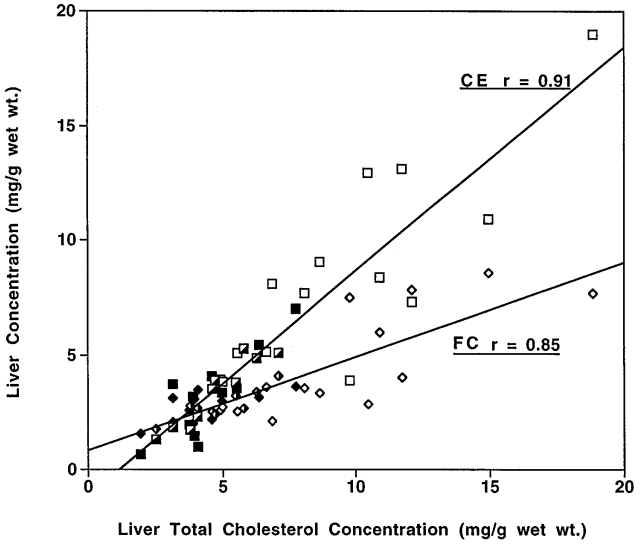

Figure 1. Comparison of liver total cholesterol concentration with unesterified (free) cholesterol (FC) and cholesteryl ester (CE) concentrations in all animals of the study. Values for each were measured in a sample of liver taken at the end of the study. Cholesteryl ester concentrations were calculated after multiplying esterified cholesterol by 1.67. Square symbols represent cholesteryl esters and diamonds represent free cholesterol. Filled symbols represent saturated fat animals, open symbols represent monounsaturated fat animals, and half-filled symbols represent polyunsaturated fat animals. The regression lines shown are for free cholesterol and cholesteryl ester for the entire dataset since regression lines for each diet group were not different; the correlations were highly significant ($P < 0.001$).

perfusion. We have used the term accumulation rate since the values were obtained during recirculating liver perfusion, but during a period of nonrecirculating perfusion that was done in a subset of these animals (Rudel, L.L., Carr, T.P, Haines, J., Sawyer, J.K., and Shah, R., manuscript in preparation), the rates were similar. Therefore, accumulation rates for recirculating perfusion probably reflect actual secretion rates. A comparison of rates of accumulation in liver perfusate among the dietary fat groups is shown in Table III. In general, the average rates of accumulation of all of the perfusate lipids were higher for the monounsaturated fat group, but the differences for each lipid were not statistically significant. Phospholipid and triacylglycerol accumulated at higher rates than cholesterol in each diet group with phospholipid accumulating at the highest rate of any lipid in all diet groups.

In spite of statistically significant diet-related differences in plasma concentrations for each of apoA-I, apoA-II, apoB, and apoE (1), no significant diet-related differences in the rate of accumulation in liver perfusate were found for any of these apolipoproteins (Table III). For both apoA-I and apoB, over 1 mg/h accumulated during perfusion. Within a diet group, the accumulation rate of apoB was generally higher than for apoA-I although the highest average value for apoA-I in the monounsaturated fat group, 1.36±0.12 mg/hg per hr, was the same as the lowest average for apoB, 1.36±0.16 mg/hg per hr, for the saturated fat group. ApoE and apoA-II appeared at slower rates, with the rate of apoA-II accumulation being only about 10–15% of that of apoA-I. Within diet groups, correlations between the plasma concentrations and the rates of accumulation in perfusate for apoA-I, apoB, and apoE were generally below $r = 0.5$, and below statistical significance. This suggests that factors controlling lipoprotein maturation are more important in determining plasma apolipoprotein concentrations.

The accumulation of individual cholesteryl esters in perfusate was then evaluated, as shown in Table IV. The diet effects were similar in degree to those shown in Table II. Cholesteryl oleate was the major liver perfusate cholesteryl ester in the monounsaturated fat group (67.3±1.6%) and in the saturated fat group (47.9±4.9%) and this percentage was significantly different among each of the diet groups. Cholesteryl linoleate was the major cholesteryl ester in the perfusate of the polyunsaturated fat group (54.6±2%), but was no more than 14% in the other groups. The difference between the polyunsaturated fat group and the other two groups was highly significant. The percentages of cholesteryl palmitate and cholesteryl stearate were highest in the liver perfusate from animals fed saturated fat but together these esters made up only about 20% of the cholesteryl ester that accumulated in this group while they were about 10% in the other diet groups. The differences were highly significant. Cholesteryl palmitoleate was about 10% or less of the perfusate cholesteryl esters of each diet group and no differences among groups was found; cholesteryl eicosenoate and arachidonate were present only in minor amounts and these values are not shown.

Relationships between liver cholesterol concentrations and plasma cholesterol concentrations were examined, as shown in Fig. 2 *A*. Significant positive correlations were seen for each diet group. The regression lines show that for the same concentration of liver cholesterol, plasma cholesterol concentrations are higher in the monkeys fed saturated fat than in those fed polyunsaturated or monounsaturated fat. The liver cholesterol concentrations in some of the animals fed monounsaturated fat reached much higher values than for animals in the other two diet groups. Similarly, the relationships between liver cholesterol concentration and plasma apoB concentrations (Fig. 2 *B*) were also well correlated and statistically signif-

Table III. Lipid and Apolipoprotein Accumulation during Liver Perfusion

		Liver perfusate accumulation rate (mg/hg per h)							
Diet	N	TG	CE	FC	PL	ApoAI	ApoAII	ApoB	ApoE
Sat	11	4.19±1.08	1.77±0.33	1.51±0.16	6.41±1.17	1.10±0.12	0.163±0.025	1.36±0.16	0.44±0.05
Mono	13	8.44±1.84	2.66±0.53	2.23±0.36	9.72±1.36	1.36±0.12	0.201±0.021	1.64±0.18	0.55±0.07
Poly	9	4.34±0.69	2.09±0.61	1.79±0.19	8.05±0.98	1.09±0.15	0.140±0.027	1.63±0.27	0.81±0.38
Significance by ANOVA		0.07	0.42	0.17	0.16	0.26	0.20	0.55	0.44

Table IV. Liver Perfusate Cholesteryl Ester Percentage Composition

Diet	*n*	Cholesteryl ester fatty acid (% wt/wt)				
		16:0	18:0	18:1	18:2	16:1
SAT	7	14.2±1.2	6.3±0.9	47.9±4.9	14.3±1.6	11.9±3.1
MONO	8	7.1±0.6	3.1±0.4	67.3±1.6	10.9±0.8	8.53±1.2
POLY	7	8.1±0.7	3.03±0.2	23.3±1.5	54.6±2.0	7.03±1.2
Significance by ANOVA (*P*)		0.0001	0.0006	0.0001	0.0001	0.24
Fisher's Post Hoc						
Sat vs Mono		0.0001	0.0006	0.0002	0.12	
Sat vs Poly		0.0001	0.0007	0.0001	0.0001	
Mono vs Poly		NS	NS	0.0001	0.0001	

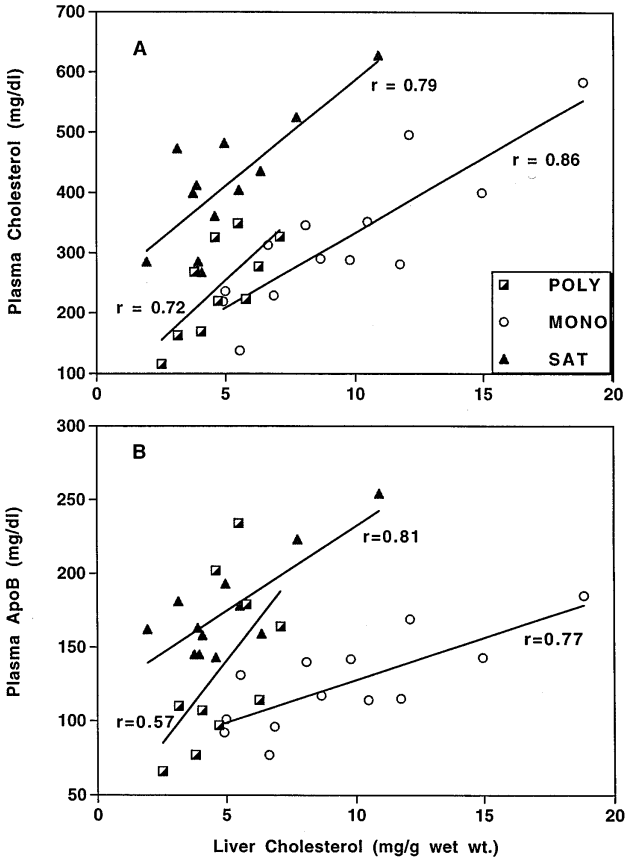

Figure 2. (*A*) Comparison of liver cholesterol concentration with plasma cholesterol concentration. Plasma cholesterol concentrations were measured in each animal periodically throughout the 5-yr study, and the values shown represent the average of these values for each animal. Liver cholesterol was measured in a sample of liver taken at the end of the study. The regression lines for each diet group are shown as are the correlation coefficients. All correlations were statistically significant ($P < 0.01$). (*B*) Comparison of liver cholesterol concentration with plasma apoB concentration. Plasma apoB concentrations were measured periodically throughout the experimental period, and the values shown are averages for each animal. Liver cholesterol was measured in a sample of liver taken at the end of the study. Regression lines are shown for each group as are correlation coefficients. The correlation for the polyunsaturated fat group was significant at $P < 0.05$, while the correlations for the other two groups were significant at the $P < 0.01$ level.

icant for each diet group and of similar magnitude to those for total plasma cholesterol.

Liver perfusion permitted direct examination of the role of hepatic cholesteryl ester secretion in plasma lipoprotein cholesterol concentrations. While the absolute rates of lipoprotein secretion during perfusion may not reflect actual in vivo rates, the proportionality between the in vitro perfusion rates and parameters of cholesterol metabolism in vivo has been repeatedly demonstrated with this liver perfusion system (14, 17, 26). Accumulation during recirculating perfusion is an accurate reflection of actual secretion rate, based on direct comparisons with accumulation rates in nonrecirculating liver perfusions [Rudel, L.L., manuscript in preparation]. Relationships between liver cholesteryl ester concentration and liver perfusate cholesteryl ester accumulation rates were examined as shown

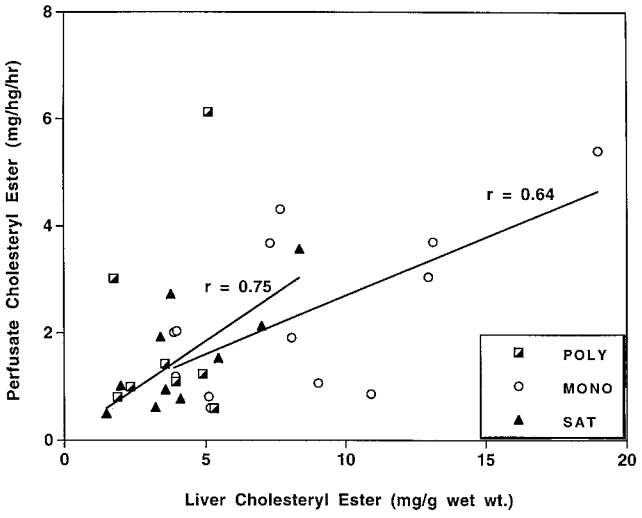

Figure 3. Comparison between the liver cholesteryl ester concentration and liver perfusate cholesteryl ester accumulation rate. Liver cholesteryl ester was measured as for Fig. 1, and liver cholesteryl ester accumulation rate was measured as the slope of the line for six timed samples of liver perfusate for each animal, and is expressed as the mg of CE per 100 g (hg) of liver per h. Each liver weighed ~ 100 g. The regression lines shown are for the data in the saturated and monounsaturated fat groups. The correlations for these relationships were statistically significant ($P < 0.01$). No significant relationship was seen for the polyunsaturated fat group.

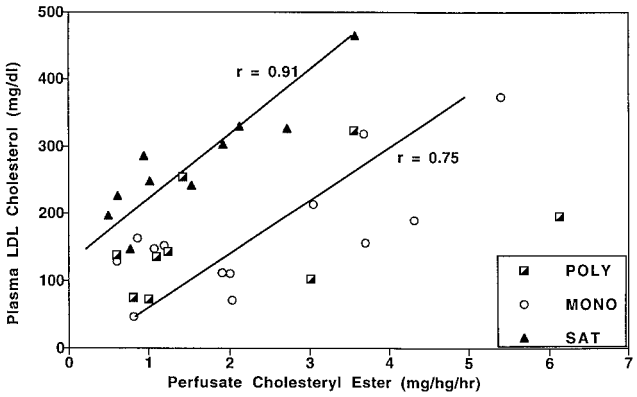

Figure 4. Comparison of liver perfusate cholesteryl ester accumulation rate and plasma LDL cholesterol concentration. Liver perfusate cholesteryl ester accumulation rate was measured as in Fig. 3, and plasma LDL cholesterol was measured periodically throughout the 5-yr experimental period. Regression lines for the saturated fat group and the monounsaturated fat group are shown and the correlation coefficients are also shown, with both being statistically significant, $P < 0.01$. The data for the polyunsaturated fat group did not define a significant correlation.

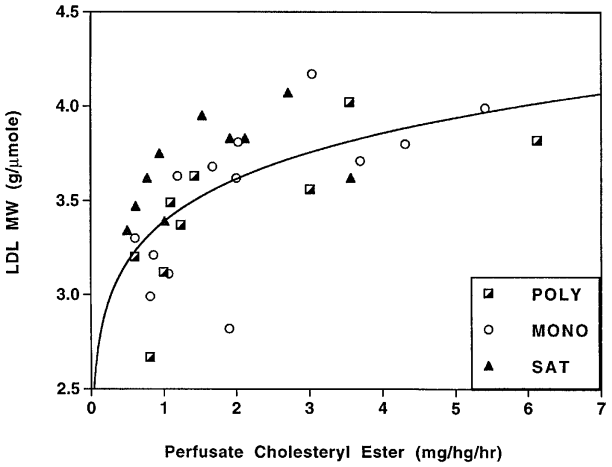

Figure 5. Comparison of liver perfusate cholesteryl ester accumulation rate and plasma LDL particle size, measured as molecular weight. The perfusate cholesteryl ester accumulation rate was measured as for Fig. 3 and the plasma LDL molecular weight was measured during gel filtration chromatography (27) and for each animal, was an average determined on samples analyzed periodically throughout the 5-yr experimental period. The regression line for the relationship between the log of the LDL molecular weight and the cholesteryl ester accumulation rate is shown, and the relationship was essentially the same for each of the diet groups ($r = 0.65$).

in Fig. 3. These endpoints were significantly correlated in the monounsaturated fat ($r = 0.64$) and saturated fat ($r = 0.75$) groups. Interestingly, in the polyunsaturated fat group, these endpoints were not well related and the correlation ($r = 0.21$) was not statistically significant. We examined the data for a relationship between liver cholesteryl ester concentration and perfusate apoB accumulation rate but no significant correlations were found for any of the diet groups.

The data in Fig. 4 show that statistically significant relationships between plasma LDL cholesterol concentrations and liver perfusate cholesteryl ester accumulation rates were found for the saturated and monounsaturated fat groups. In the monounsaturated fat group, more hepatic cholesteryl ester secretion is required to maintain LDL cholesterol concentrations equivalent to those in the saturated fat group. The polyunsaturated fat group may have been more similar to the monounsaturated fat group although the data do not define a significant relationship. In contrast, a significant correlation was identified between liver perfusate cholesteryl ester accumulation rate and plasma apoB concentration only for the saturated fat group, $r = 0.6$ (data not shown). Correlations in the monounsaturated fat and polyunsaturated fat group were not significant. Cholesteryl ester secretion by the liver, as a result of liver cholesteryl ester enrichment, appears more important in determining plasma LDL cholesterol concentration than apoB concentration, i.e., the effect of the liver to increase plasma LDL cholesterol concentration is more through cholesteryl ester enrichment of the particles than in increasing the number of particles.

The relationship between liver perfusate cholesteryl ester accumulation rate and plasma LDL particle size (principally a measure of cholesteryl ester enrichment of the particle [27]) is shown in Fig. 5. A statistically significant relationship, indistinguishable among diet groups in both degree and pattern of association, was found. The data described a highly significant ($P < 0.001$) logarithmic relationship and the regression line is shown. As perfusate cholesteryl ester accumulation rates increased, LDL particles became larger, but for larger LDL particle sizes, further increases in cholesteryl ester accumulation led to decreasingly smaller increments in LDL particle size.

In addition to the relationship to particle size, liver cholesteryl ester secretion, measured by accumulation rate during recirculating perfusion, was significantly ($P < 0.05$) correlated to apoB accumulation rate in perfusate in the monounsaturated and saturated fat groups (Fig. 6). While the data for the livers

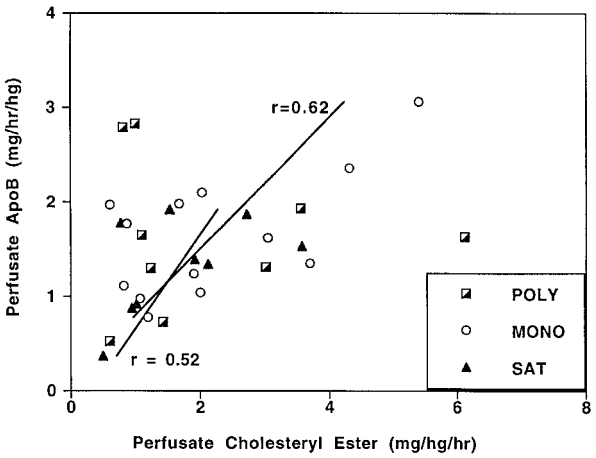

Figure 6. Comparison of liver perfusate cholesteryl ester accumulation rate and perfusate apoB accumulation rate. Perfusate cholesteryl ester accumulation rate was measured as in Fig. 3 and perfusate apoB accumulation rate was measured by ELISA in six timed perfusate samples, and represents the slope of the line for each animal in each diet group. Significant relationships in the saturated fat group ($r = 0.52$, $P < 0.05$) and in the monounsaturated fat group ($r = 0.62$, $P < 0.05$) defined essentially the same regression line. No significant relationship was seen in the polyunsaturated fat group.

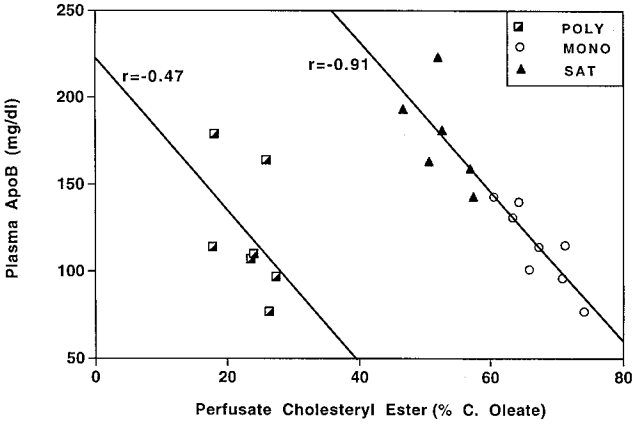

Figure 7. Comparison of the percentage of perfusate cholesteryl ester as cholesteryl oleate with plasma apoB concentration. Plasma apoB concentration was measured for each animal as described for Fig. 2 *B* and the perfusate cholesteryl ester composition was measured after lipid extraction of perfusate lipoproteins, separation of cholesteryl esters by TLC, and quantitation of individual cholesteryl ester fatty acids by GLC (1). The relationships for the saturated and monounsaturated fat groups were defined by essentially the same regression line and the correlation coefficient for this line is shown ($r = 0.91$, $P < 0.001$). The relationship ($r = 0.47$) for the polyunsaturated fat group was not statistically significant at the $P = 0.05$ level, but the slope was the same as for the other two groups, therefore the line was shown.

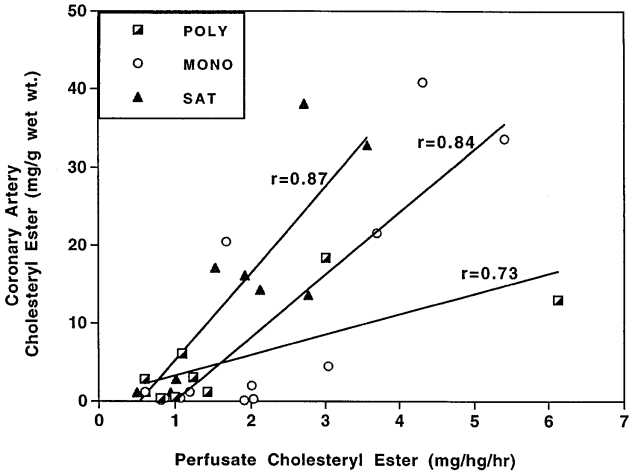

Figure 8. Comparison of liver perfusate cholesteryl ester accumulation rate and coronary artery cholesteryl ester concentration, as a measure of the extent of coronary artery atherosclerosis. Coronary artery cholesteryl ester concentration was measured after isolation and lipid extraction of the left anterior descending coronary artery (1). The correlations for each diet group were statistically significant ($P < 0.01$ for SAT and MONO and $P < 0.05$ for POLY) although the relationship was different for each diet group.

of animals fed polyunsaturated fat do not describe a significant relationship, many of these data points appear to fit a similar relationship to that of the other two groups. In general, it appears that when cholesteryl ester secretion is higher, apoB secretion is also higher, but that this association is dependent on dietary fat type.

A relationship between plasma apoB and the type of cholesteryl ester secreted, monitored as the percentage of cholesteryl oleate in perfusate, was found as shown in Fig. 7. A strong correlation ($r = -0.9$, $P < 0.001$) was obtained for this comparison in the saturated and monounsaturated fat groups. The data for each diet group define a distinct area in the graph, but the slopes of each of the three regression lines were similar, as were the *y*-intercepts for the two lines for the saturated fat and monounsaturated fat groups. This relationship indicates that the degree of cholesteryl oleate enrichment of the apoB-particles secreted by the liver is actually very important in determining the final concentration of apoB in the plasma of these animals. The apoB values for each animal are average values that were derived from periodic measurements taken throughout the five-year experimental period, thus the strength of this relationship was felt to be even more important, since the percentage of cholesteryl oleate in perfusate appears to be reporting on a long-term regulation of plasma apoB concentration.

Significant relationships have been identified between hepatic cholesteryl ester secretion and cholesteryl ester enrichment of plasma LDL, which, in turn has been shown previously to be predictive of the extent of coronary artery atherosclerosis (15, 28–31). A direct comparison of hepatic cholesteryl ester secretion rate to coronary artery atherosclerosis was made. Liver cholesteryl ester accumulation during perfusion, as an estimate of hepatic cholesteryl ester secretion rate in vivo, was compared to cholesteryl ester concentration in the left anterior descending coronary artery, as shown in Fig. 8. Statistically significant relationships were found for each diet group, with the regression line relationships for each group being different. The strongest relationship was found in the saturated fat group, where the correlation coefficient was $r = 0.87$, $P < 0.01$. The rate of cholesteryl ester accumulation was somewhat higher in the monounsaturated fat group so that the regression line was shifted to the left, but the rate of cholesteryl ester accumulation during isolated perfusion remained a surprisingly accurate estimate ($r = 0.84$, $P < 0.01$) of the amount of cholesteryl ester that would accumulate in the coronary artery during the five years of diet induction of atherosclerosis. The relationship was also significant for the polyunsaturated fat group, although the correlation coefficient was smaller for this group, $r = 0.73$, $P = 0.01$. A comparison of the regression analysis shows that at any of the higher rates of hepatic cholesteryl ester secretion, e.g., 3 mg/hg per h, the amount of coronary artery atherosclerosis was highest in the saturated fat group, next highest in the monounsaturated fat group, and least in the polyunsaturated fat group.

The correlations between coronary artery cholesteryl ester content and plasma lipoprotein endpoints identified previously (1) were generally not as high as the correlations with hepatic cholesteryl ester secretion for any of the diet groups, although many of the lipoprotein correlations were statistically significant. This outcome suggests that hepatic secretion of cholesteryl esters, which were predominantly cholesteryl oleate in the groups with the most atherosclerosis, may promote atherosclerosis through a mechanism such as cholesteryl oleate enrichment of a spectrum of lipoprotein particles that subsequently interact with and cause cholesteryl ester deposition in the wall of the coronary artery. The fact that dietary fat of different compositions shifted the relationship between hepatic cholesteryl ester secretion and atherosclerosis, indicated that

dietary fat type importantly alters atherogenesis at least in part through effects on liver cholesteryl esters in this monkey model of experimental atherosclerosis.

Discussion

The data in this manuscript address the question of whether a diet-induced increase in availability of hepatic cholesteryl oleate results in an enrichment of cholesteryl oleate in plasma lipoproteins causing coronary artery atherosclerosis in African green monkeys, a primate model of experimental atherosclerosis. The question was identified as important when it was found that a diet rich in oleate promoted a marked cholesteryl oleate enrichment of lipoproteins and, in spite of lower LDL cholesterol and higher HDL cholesterol, failed to protect against coronary artery atherosclerosis.

A detailed analysis of liver lipid composition and of lipid and lipoprotein secretion during isolated liver perfusion was made, and outcomes were compared to plasma lipoprotein and coronary artery atherosclerosis endpoints using correlation and regression analyses. The first aspect that became clear was that a diet enriched in oleate promotes free and esterified cholesterol deposition in the liver. The livers of the monounsaturated fat group compared to those of the other diet groups contained more than twice the concentration of cholesteryl ester, of which over 74% was cholesteryl oleate (Tables I and II). The amount of liver cholesteryl ester that accumulated was positively correlated to higher plasma cholesterol concentrations (Fig. 2) and to higher secretion rates of cholesteryl ester during isolated liver perfusion (Fig. 3), suggesting that lipoproteins enriched in cholesteryl oleate by the liver were the source of the cholesteryl oleate-enriched plasma LDL. Plasma LDL particle size, as a measure of cholesteryl ester-enrichment of LDL particles, was larger in the saturated and monounsaturated fat groups (1) and particle size correlated well with cholesteryl ester accumulation rate in perfusate (Fig. 5).

Cholesteryl ester secretion by the liver also was significantly correlated to apoB secretion in two of the three diet groups, specifically the saturated and monounsaturated fat groups in which cholesteryl oleate predominates (Fig. 6). In an earlier publication (20) the secretion of apoB was tied to cholesteryl ester secretion by the liver, and this is another case in which this association is apparent. It may be that cholesteryl oleate, specifically, is the more important cholesteryl ester and somehow facilitates this association. The degree to which the perfusate cholesteryl ester was enriched in cholesteryl oleate, measured as percentage, was strongly and inversely related to plasma apoB concentration (Fig. 7). The strength of this association was notable ($r = 0.9$), especially when no significant association between plasma apoB concentration and perfusate cholesteryl ester accumulation rate was seen. The majority of plasma apoB in these animals is in the LDL fraction. The inverse relationship of Fig. 7 suggests that the formation of LDL in the circulation from apoB-containing hepatic lipoproteins is a function of the degree of cholesteryl oleate enrichment.

To the extent that cholesteryl esters are present primarily in neutral lipid core-containing lipoproteins, the bulk of the perfusate cholesteryl esters are in apoB-containing lipoproteins, since the HDL in perfusate are discoidal, phospholipid-rich, and neutral lipid-poor lipoproteins (26, 32, 33). Since LCAT is relatively inactive in perfusate (33), most of the cholesteryl ester in perfusate appears to have been derived from hepatic ACAT, and the fatty acid composition of perfusate cholesteryl esters reflects that of tissue cholesteryl esters (Tables II and IV). The inclusion of ACAT-derived cholesteryl esters in perfusate lipoproteins presumably occurs through their secretion in apoB-containing lipoproteins (20), although we have not ruled out the possibility that some cholesteryl ester in recirculating liver perfusate could have been derived from plasma membranes of hepatocytes through CETP-mediated exchange, for example. Immunoaffinity isolation of apoB-containing lipoproteins and documentation of their cholesteryl ester content was not done, but the bulk of the cholesteryl ester and apoB was isolated from perfusate in the $d < 1.063$ density range, and the material in this density range, while very heterogeneous, is almost all lipoprotein material containing apoB (17, 20, 26).

Increased hepatic secretion of cholesteryl oleate appears to be associated with more cholesteryl ester per apoB particle and we assume that the liver secreted a greater proportion of apoB-containing particles with too many cholesteryl ester molecules to remain in plasma as LDL. Particle compositions of perfusate VLDL were defined previously (17, 20). Many of the cholesteryl ester-enriched VLDL particles presumably rapidly became cholesteryl ester-enriched remnant particles in the circulation and were cleared as remnants resulting in lower apoB-particle concentrations in plasma, rather than becoming LDL particles (34). Rapid conversion of a portion of perfusate VLDL into LDL in the circulation was also described in this earlier study, and presumably in the monounsaturated fat-fed animals, the proportion undergoing this conversion would be more limited, although this was not specifically measured. The limiting factor may be the high number of cholesteryl ester molecules per apoB molecule. It is also possible that apoB in particles with increased cholesteryl oleate assumes a different conformation that affects clearance although precedent for this is not established.

Our findings are generally consistent with those of Dietschy and colleagues (35, 36) although hepatic secretion of cholesteryl ester is highlighted more by our data. Liver cholesteryl ester concentrations were higher in the monkeys fed monounsaturated fat compared to saturated fat, a finding similar to that found in hamsters (36). The fact that there were generally higher apoB concentrations in the plasma of the saturated fat animals may be an indication that hepatic LDL receptor levels in this group are generally lower and more downregulated by dietary cholesterol than in the monounsaturated fat group, as has been suggested to occur in other species (35). However, we identified a positive relationship between hepatic cholesteryl ester concentration, cholesteryl ester secretion, and plasma LDL cholesterol concentration (Figs. 3 and 4) where a trend was seen for the amount of cholesteryl ester secreted by the liver to be higher in the monounsaturated fat group. Cholesterol production by the liver was not quantitated in hamsters, but the data implied that LDL cholesterol production rate was lower when oleic acid was fed than when saturated fatty acids were fed (36). The strong inverse association between plasma apoB concentration and the percentage of cholesteryl oleate in secreted cholesteryl esters, as shown in Fig. 7 for the saturated and monounsaturated fat groups, suggests that one of the primary factors controlling plasma cholesterol is related to the composition (and subsequent metabolism) of secreted lipoproteins in primates and is not solely LDL receptor function, as has been assumed to be the case in hamsters (35, 36). Never-

theless, the role of the LDL receptor in the liver in clearance of VLDL and VLDL remnants, before their incorporation into LDL, may be important, although it was not measured in our monkey studies.

An important role for hepatic ACAT in cholesteryl ester accumulation in the liver, in LDL enrichment with cholesteryl esters, and in coronary artery atherosclerosis in African green monkeys has been previously identified and described (14). The data of the present paper extend these observations into monkeys fed oleate-rich diets. Oleate enrichment of hepatic and plasma pools of cholesteryl esters, which was marked when saturated fat and cholesterol was fed, was greatly enhanced when monounsaturated fat and cholesterol was fed. The possibility that oleate is an activator of ACAT is suggested when one considers that liver cholesteryl ester concentrations (75% of which is cholesteryl oleate) in the monounsaturated fat animals are more than twofold higher than when saturated or polyunsaturated fat was fed (Tables I and II). The suggestion that oleate activates ACAT is not new but was originally demonstrated by the work of Goodman et al. (16) in 1964. From the data on liver cholesteryl ester composition and concentration in the polyunsaturated fat group, it is apparent that linoleate can be esterified to cholesterol by ACAT when this fatty acid is predominant in the diet. If one considers the earlier work in rat liver microsomes where linoleate was incorporated least well from a mixture of acylCoAs (16), these observations suggest that the enrichment of linoleyl CoA in the acylCoA pool must have been considerable in our monkeys. The fact that the amount of cholesteryl ester that accumulates in the liver (Table I) and gets secreted by the liver (Table III) is the same when polyunsaturated fat and saturated fat were fed suggests that when abundant in the acylCoA pool, however, linoleyl CoA is effectively utilized by ACAT for cholesteryl ester formation.

The observation in these monkeys that the amount of coronary artery atherosclerosis was similar in the monounsaturated and saturated fat groups, in spite of the significantly improved LDL cholesterol concentration and LDL/HDL cholesterol ratio in the former (1), suggested that the presence of increased amounts of cholesteryl oleate that appear to originate from liver ACAT and accumulate in plasma LDL was very proatherogenic and overcame the improvement in LDL and HDL cholesterol concentrations. When oleate is the major dietary fatty acid, a similar extent of enrichment of cholesteryl esters with cholesteryl oleate occurred in the liver (Table II), in liver-secreted lipoproteins (Table IV), in plasma lipoproteins (1), and in the coronary arteries (1). Increased amounts of cholesteryl oleate proportional to an increase in particle size of plasma LDL in monkeys have consistently been correlated to increased amounts of coronary artery atherosclerosis in several previous nonhuman primate studies (15, 28–31). In several of these studies, polyunsaturated fat has been shown to protect against atherosclerosis, and this could be due to the fact that in animals fed polyunsaturated fat, accumulation of cholesteryl oleate in lipoproteins is blocked.

The importance of cholesteryl oleate enrichment of lipoproteins in promoting atherogenesis appears to have relevance to coronary heart disease in humans since some similarities are seen. Increased amounts of cholesteryl oleate in plasma LDL mean decreased amounts of cholesteryl linoleate (10, 37), since these are the two major cholesteryl esters in plasma. Several studies have showed an inverse relationship between the percentage of cholesteryl linoleate in plasma and the complications of atherosclerosis in human patients (9–13, 38–41). In this context, a curious situation has occurred where some investigators have measured an increase in the percentage of cholesteryl oleate in LDL (and a decrease in the percentage of cholesteryl linoleate) when diets rich in monounsaturated fat were fed and, at the same time, have recommended diets rich in oleic acid to protect against heart disease because in the test tube, oleate-rich LDL oxidize less well upon incubation with copper (37). The numerous observations showing that more cholesteryl linoleate is associated with decreased CHD risk suggest that recommendations based on test tube oxidation reactions may not be as well placed as observations related to the disease endpoint itself.

The mechanism through which oleate would promote atherogenesis is a matter for speculation. An increased percentage of cholesteryl linoleate may serve to maintain a more liquid phase in the core lipids of LDL (1) and in the lipid droplets that form during lesion development, thereby facilitating cholesteryl ester mobilization and/or transfer (42, 43). The surface properties of the lipoproteins may be different, so that the interactions of the lipoproteins are altered. For example, we have found that the interaction of LDL with proteoglycans of the artery wall was facilitated by dietary monounsaturated fat (44), so that retention of LDL in the artery wall may occur to a greater extent in the monounsaturated fat group. LDL oxidation, and any putative role for this phenomenon in atherosclerosis, would not appear to be part of the explanation. LDL with increased proportions of cholesteryl oleate and decreased proportions of cholesteryl linoleate, as occurs when oleic acid-rich diets are fed, has a decreased susceptibility to oxidation when compared to LDL isolated from animals fed a polyunsaturated fat diet (19). In contrast, the amount of coronary artery atherosclerosis was less when polyunsaturated fat was fed than when monounsaturated fat was fed (1). The whole animal data on coronary artery atherosclerosis outcome therefore indicate that cholesteryl oleate enrichment of lipoprotein particles is probably more proatherogenic in monkeys than the potential for LDL oxidation.

In summary, the liver appears to be the primary source of the cholesteryl oleate that accumulates in plasma LDL particles. The cholesteryl oleate enrichment occurs as a result of the hepatic secretion of ACAT-derived cholesteryl oleate in apoB-containing lipoproteins. The increased secretion of cholesteryl oleate enriched lipoproteins by the liver was well correlated with the extent of coronary artery atherosclerosis in these African green monkeys.

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