Peritoneal Macrophage Cholesteryl Ester Content as a Function of Plasma Cholesterol in Rats

Roberto Musanti, Augusto Chiari, and Giancarlo Ghiselli

Cholesterol accumulation in macrophages that have migrated in the subintimal space leads to foam cell formation, which is believed to be one of the initiating events in atherosclerosis. In this study we investigated the effect of cholesterol feeding on peritoneal monocyte/macrophage cholesterol content and peritoneal cavity lipoprotein composition in rats. A cholesterol (2%) and cholic acid (1%) diet caused significant hypercholesterolemia in plasma, and at the same time the cholesterol content of peritoneal monocytes/macrophages was increased. At day 7, the cellular cholesteryl ester content had risen to 30.1 μg/mg cellular protein from a baseline value of 9.2 μg/mg. The esterified cholesterol content also increased by 56%. At this time, acyl-coenzyme A:cholesterol acyltransferase (ACAT) activity was doubled, whereas neutral and acidic cholesteryl ester hydrolase activities were unchanged. Reversal to the regular chow diet after 7 days of the cholesterol-enriched diet normalized plasma cholesterol levels as well as peritoneal monocyte/macrophage cholesteryl ester content. ACAT activity also decreased toward normal levels. Analysis of the d<1.21 g/ml peritoneal lipoproteins isolated by ultracentrifugation revealed the presence, in both normal and hypercholesterolemic rats, of apolipoprotein A-I-rich lipid complexes with pre-β mobility on agarose gel electrophoresis. The size of the peritoneal lipoproteins was smaller than that of plasmatic high density lipoproteins, and their chemical composition was also different from that of the major plasma lipoproteins. The cholesteryl ester content of peritoneal lipoproteins increased after feeding of the cholesterol-enriched diet. In conclusion, our results show that cholesterol feeding leads to rapid accumulation of cholesteryl esters in monocytes/macrophages. As soon as plasma cholesterol levels are returned to normal, cellular cholesterol content is also normalized. This finding is in accordance with the concept that cholesterol homeostasis in monocytes/macrophages is influenced by the level of circulating lipoproteins. Lipoproteins isolated from the peritoneal cavity may be the same as the pre-β-migrating apolipoprotein A-I lipoproteins identified in plasma that are involved in the early phases of reverse cholesterol transport. (Arteriosclerosis and Thrombosis 1991;11:1111–1119)
Although the process leading to lipoprotein internalization by macrophages has been well investigated in vitro, little is known about the process in vivo. Prior studies on the effect of plasma cholesterol elevation on macrophages through dietary manipulation have mainly focused on some selected functional changes that rat peritoneal macrophages may undergo.\textsuperscript{12,13} In all cases, cholesterol feeding before cellular harvesting was lengthy, lasting at least several weeks. In this study the effect of repeated cholesterol feeding on cholesterol accumulation in peritoneal macrophages has been investigated. The time course of cholesterol accumulation and of cholesterol efflux after reversal of diet-induced hypercholesterolemia by feeding of a normal diet has been examined. Furthermore, the compositional and structural properties of peritoneal fluid lipoproteins have been analyzed.

Methods

Animals

Male Sprague-Dawley rats (Charles River Lab) weighing 200–225 g were used throughout the experiments. Animals were housed under 12-hour light/dark conditions and had free access to food and water. Animals were fed either regular rat chow or a cholesterol-enriched diet consisting of regular rat chow supplemented with 2% cholesterol and 1% cholic acid. The cholesterol-enriched diet was prepared by an outside manufacturer (Piccioni, Milan, Italy), and cholesterol and cholic acid were added to the diet, mixed as powders before extrusion through the pelleting machine.

Peritoneal Macrophage Preparation

Macrophages were obtained by peritoneal washing as follows. Fasted animals were anesthetized with ethyl ether, and 20 ml phosphate buffer was infused into the peritoneal cavity. The abdomen was gently massaged, and 15–17 ml of the fluid was aspirated with a syringe. Cells were pelleted by centrifugation at 800g for 10 minutes in a refrigerated centrifuge. Preparations in which red blood cells were present were discarded. Collected peritoneal cells were resuspended in a mixture of minimal essential medium–10% fetal calf serum and plated in plastic dishes. After 3 hours' incubation in a 5% CO\textsubscript{2}/95% air incubator at 37°C, nonadherent cells were washed out with three subsequent additions of phosphate buffer. Adherent macrophages were scraped off with a rubber policeman and used immediately or stored at -80°C. Cells were visualized on a Leitz Diavert light microscope at x128 magnification.

Peritoneal Fluid

Peritoneal cavity washings from three to five rats were pooled, the volume was measured, and the fluid was concentrated in 12,000 molecular weight cutoff dialysis tubing in polyethylene glycol 6000. The concentrated material was dialyzed overnight against 0.15 M NaCl, 10 mM tris(hydroxymethyl)aminomethane (Tris) HCl, and 0.01% NaN\textsubscript{3} (pH 7.4), and then the background density was raised to 1.21 g/ml by addition of a calculated amount of KBr. The concentrated peritoneal fluid was subjected to ultracentrifugation in a 60Ti Beckman rotor spinning at 40,000 rpm for 24 hours. The top (the upper 5 ml), the middle (middle 10 ml), and the bottom (lower 10 ml) fractions were collected separately, dialyzed against Tris buffer, and concentrated with polyethylene glycol 6000 as detailed above. Cholesterol (unesterified and total) was measured by an enzymatic fluorometric technique\textsuperscript{14}; triglyceride and choline content—the latter as a measure of total phospholipid content—were determined by automated enzymatic colorimetric assays on a Coulter CPA autoanalyzer.\textsuperscript{15,16} Proteins were measured by the Lowry technique by use of a calibrated bovine serum albumin standard from Bio-Rad.\textsuperscript{17} Agarose gel electrophoresis was performed on Beckman Paragon strips, following the manufacturer's guidelines. Lipoproteins were stained with Sudan black. Human and rat plasma lipoproteins (VLDL=d<1.019 g/ml, LDL=d=1.019–1.063 g/ml, and high density lipoprotein [HDL]=d=1.063–1.210 g/ml) isolated by ultracentrifugation were used to standardize the methodology as well as to act as markers for migration in each electrophoretic run. Lipoprotein polyacrylamide gel electrophoresis under native conditions was performed in a Pharmacia FAST system with Pharmacia precast 4–15% gradient polyacrylamide gel strips. Rat plasma HDL (d=1.063–1.210 g/ml from ultracentrifugation) and purified rat plasma apolipoprotein (apo) A-I were used as migration standards. After electrophoresis, gels were stained with Coomassie blue G-250. Polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate of lipoproteins was performed as previously described with the use of 12% polyacrylamide gels.\textsuperscript{18,19} For this purpose, lipoproteins were lyophilized, and the lipid was extracted with CHCl\textsubscript{3}/CH\textsubscript{3}OH.\textsuperscript{18} Coomassie blue G-250–stained apolipoprotein bands were identified based on their apparent molecular weight and comparison with molecular weight standards. Purified rat apo A-I, apo E, and apo A-IV were also used to identify peritoneal fluid apolipoproteins.

Peritoneal Macrophage Acyl-Coenzyme A: Cholesterol Acyltransferase Activity Assay

Adherent macrophages were scraped off with a rubber policeman. Cells were homogenized in a glass–Teflon hand homogenizer in 0.1 M phosphate buffer (pH 7.4) containing 0.01% β-mercaptoethanol. The homogenate was centrifuged at 11,500g for 15 minutes in a refrigerated centrifuge, and the supernatant was collected and centrifuged at 105,000g for 1 hour. The microsomal pellet was resuspended in the homogenization buffer and re-centrifuged at 105,000g. All the operations were performed at 4°C. The resulting pellet was overlayed with the homogenization buffer and stored at -80°C until assay of acyl-coenzyme A:cholesterol acyltransferase (ACAT) enzymatic activity. The ACAT activity assay was performed essentially as described by Bell.\textsuperscript{20} The microsomal pellet was resus-
mented by several passages through a hypodermic needle, and protein content was measured according to the method of Lowry et al.\textsuperscript{17} An aliquot of resuspended microsomes was extracted for determination of cholesterol content. The ACAT assay mixture contained 0.05 mg microsomal protein, 400,000 cpm carbon-14-labeled oleyl-CoA in a 0.1 M phosphate buffer, and 0.01% \( \beta \)-mercaptoethanol (pH 7.4). Incubation was at 37°C for 15 minutes. The reaction was stopped by addition of 2 ml CH\(_3\)OH. Microsomal lipids were extracted according to Folch et al.\textsuperscript{21} after addition of tritiated cholesteryl oleate as an internal standard. The organic phase was evaporated, and lipids were separated into phospholipids, cholesterol, triglycerides, and cholesteryl esters by thin-layer chromatography with the use of precoated silica gel 60 F/254 plastic sheets from Merck. The developing system consisted of hexane/ethyl ether/ acetic acid, 180:20:2 (vol/vol/vol). Lipids were evidenced with 8% phosphomolybdic acid, and the bands were cut and counted. ACAT activity was expressed as picomoles oleate formed per hour per milligram microsomal protein.

**Peritoneal Macrophage Acidic and Neutral Cholesteryl Ester Hydrolase Activity Assay**

The 105,000-molecular-weight supernatant of the macrophage homogenate was used as a source of acidic cholesteryl ester hydrolase (ACEH) and neutral cholesteryl ester hydrolase (NCEH) activities. Enzymes were assayed essentially as described by Brecher et al.\textsuperscript{22} Cytosolic fraction proteins were determined according to Lowry et al.\textsuperscript{17} The assay mixture contained 0.1 mg cytosolic proteins, 50,000 cpm [\( ^{14} \)C]cholesteryl oleate in 0.1 M NaH\(_2\)PO\(_4\), and 0.01% \( \beta \)-mercaptoethanol. For ACEH, pH was adjusted to 5.0. For NCEH, pH was 8.0. The cocktail was incubated at 37°C for 60 minutes, and the reaction was stopped by addition of benzene/CH\(_3\)Cl/CH\(_3\)OH, 5:1:1 (vol/vol/vol) containing 0.1 mM oleic acid added as a carrier. Fatty acids were extracted after addition of 0.3 M NaOH. Samples were centrifuged, and an aliquot of the organic phase was counted. CEH activities are given as picomoles oleic acid formed per hour per milligram cytosolic protein.
Figure 2. Light-microscopic photomicrographs of peritoneal monocytes/macrophages obtained from rats fed a regular diet (panel A) or a cholesterol-enriched diet (panel B) for 1 week. Arrows point to lipid-loaded monocytes/macrophages. Magnification, ×80.

Cellular Cholesterol Content Determination

Adherent macrophages were lipid extracted in situ. Aliquots of the lipids extracted were used for the enzymatic fluorometric determination of unesterified and total cholesterol.

Blood Lipids

Blood samples were taken from animals fasted overnight into tubes containing Na₂EDTA as an anticoagulant. Plasma lipids were measured by automated techniques on a Coulter CPA autoanalyzer.

Results

Cholesterol feeding brought about massive hypercholesterolemia in the rats, reaching a maximum (352±46 mg/dl) between the seventh and the 14th day after beginning the diet (Figure 1A). Afterward there was a decline in plasma cholesterol concentration, which adjusted to a lower level after 1–2 months of the diet (between 200 and 230 mg/dl). The changes in peritoneal macrophage cholesterol content behavior were found to mirror closely those in plasma cholesterol concentration (Figure 1B). Thus, macrophage cholesterol content reached a maximum between the third and the 14th day after beginning the cholesterol-enriched diet. After the second week the concentration decreased, and about the fourth week intracellular cholesterol content adjusted to a level lower than that observed at 2 weeks (48.3 versus 67.5 μg cholesterol/mg cell protein) yet was still significantly higher than that observed before cholesterol feeding (28.7 μg cholesterol/mg cell protein).

When peritoneal macrophage content was analyzed for unesterified and esterified cholesterol, as much as a fourfold elevation in cholesteryl esters was detected. In particular, cellular cholesteryl ester content reached its peak around the seventh day of the manipulated diet (28.3 versus 6.1 μg/mg cell protein at the beginning) and remained very high until the end of the observation period (20.1 and 18.7 μg/mg cell protein at 2 and 8 weeks, respectively). Unesterified cholesterol content also increased in response to the diet, reaching 47.1 μg/mg cell protein at 2 weeks (22.8 μg/mg before the diet). Although it decreased
with time, it was still elevated at 8 weeks (31.1 μg/mg cell protein). Consistent with the measured cellular cholesterol content, light-microscopic examination of macrophages isolated after 1 week of the cholesterol-enriched diet revealed the presence of lipid vacuoles in the cytoplasm not present in the macrophages of animals fed the normal diet (Figure 2).

A new experimental dietary schedule was adopted to determine the time course of plasma and cellular cholesterol decreases after cessation of the cholesterol diet. In this experiment animals received a cholesterol-enriched diet for as long as 1 week and were subsequently returned to the regular rat chow diet for 7 days. Plasma and cellular cholesterol concentrations were measured before the diet and at 3, 7, 10, and 14 days thereafter, with animal sacrifice occurring at selected times for different groups of animals. The results are presented in Figure 3, which also illustrates the dietary schedule. Feeding of the cholesterol diet caused the expected plasma and peritoneal monocyte/macrophage total cholesterol content to rise, and the changes were already significant at the third day. By the seventh day, plasma cholesterol concentration was 369 ± 70 mg/dl (70±13 mg/dl before the diet). Macrophage esterified cholesterol content at this time was 30.1 μg/mg cell protein (9.2 μg/mg before diet), and unesterified cholesterol was 37.2 μg/mg cell protein (24.7 μg/mg

<table>
<thead>
<tr>
<th>Animal groups</th>
<th>ACAT activity (pmol cholesteryl oleate formed/mg microsomal protein/hr)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal diet</td>
<td>357±8</td>
</tr>
<tr>
<td>HC diet for 3 days</td>
<td>737±18*</td>
</tr>
<tr>
<td>HC diet for 7 days</td>
<td>677±36*</td>
</tr>
<tr>
<td>HC diet, normal diet for 7 days each</td>
<td>489±40*</td>
</tr>
</tbody>
</table>

Each value is mean±SD from triplicate determinations from a single pooled cell preparation form 10 rats.

ACAT, Acyl coenzyme A:cholesterol acyltransferase; HC, high-cholesterol.

*p<0.01 versus normal diet group.
TABLE 2. Acid and Neutral Cholesteryl Ester Hydrolase Activity in Cytosols From Peritoneal Macrophages From Rats Fed a Normal, a Hypercholesterolemic, or a Hypercholesterolemic Followed by a Normal Diet

<table>
<thead>
<tr>
<th>Animal groups</th>
<th>ACEH (nmol oleate/mg protein/hr)</th>
<th>NCEH (pmol oleate/mg protein/hr)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal diet</td>
<td>12.0±0.07</td>
<td>9.5±1.2</td>
</tr>
<tr>
<td>HC diet for 3 days</td>
<td>10.5±0.05</td>
<td>7.5±1.9</td>
</tr>
<tr>
<td>HC diet for 7 days</td>
<td>11.0±0.10</td>
<td>8.0±0.8</td>
</tr>
<tr>
<td>HC diet, normal diet for 7 days each</td>
<td>9.3±0.10</td>
<td>10.6±0.2</td>
</tr>
</tbody>
</table>

Each value is the mean±SD from triplicate determinations from a single pooled cell preparation from 10 rats.

ACEH, acidic cholesteryl ester hydrolase; NCEH, neutral cholesteryl ester hydrolase; HC, hypercholesterolemic.

In view of the marked changes elicited by diet on the cholesterol content in peritoneal monocytes/macrophages, peritoneal cavity fluid lipoprotein content was also analyzed. All the lipids were virtually recovered in the ultracentrifuge supernatant. Peritoneal cholesterol content increased from 0.6 mg/rat to 5.0 mg/rat after 1 week of the cholesterol-enriched diet. It decreased to 1.1 mg/rat by switching the diet back to normal. Lipoproteins isolated from peritoneal fluid 7 days after cholesterol feeding were analyzed for chemical composition. Results are reported in Table 3, together with the chemical composition of plasma lipoproteins sampled at the same time. Compared with peritoneal fluid lipoproteins from animals fed regular chow, those isolated from animals after 1 week of the cholesterol enriched-diet were significantly enriched in cholesteryl esters, to the detriment of the protein content. The composition of peritoneal fluid lipoproteins was significantly different from that of any of the major classes of plasma lipoproteins. In agarose gel, peritoneal fluid lipoproteins both from 1-week cholesterol-treated animals and from animals on the regular diet migrated in a similar way in a pre-β position (Figure 4). The analysis of the apolipoprotein content revealed the presence in both lipoprotein preparations of apo A-I as well as apo E and traces of apo A-IV (Figure 5), with no apparent major changes in their relative proportions as a result of cholesterol feeding. When subjected to nondenaturing gradient polyacrylamide gel electrophoresis (Figure 6), the molecular size of the peritoneal lipoproteins isolated from rats fed regular diet or the cholesterol-enriched diet for 1 week appeared smaller than that of plasmatic HDL. The size was, however, larger than that of delipidated apo A-I, consistent with their complex particle nature. The range of their size distribution was narrower compared with that of plasma lipoproteins.

Discussion

Cholesteryl ester accumulation in macrophages leads to the formation of foam cells, and this process is believed to be a key event in the early phases of the atherosclerotic process. Different authors have now observed that in pigs, rats, and monkeys experimental hypercholesterolemia leads to enhanced monocyte adherence to the arterial wall endothelium. These cells, which later migrate suben-
TABLE 3. Percent Protein and Lipid Composition of Plasma Lipoproteins and \( d < 1.21 \) g/ml Lipoproteins of Peritoneal Fluid From Rats Fed a Regular or a Cholesterol-Enriched Diet for 1 Week

<table>
<thead>
<tr>
<th>Animal groups</th>
<th>Plasma VLDL</th>
<th>LDL</th>
<th>HDL</th>
<th>Peritoneal fluid (( d &lt; 1.21 ) g/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Chol EC TG PL Pr</td>
<td>Chol EC TG PL Pr</td>
<td>Chol EC TG PL Pr</td>
<td>Chol EC TG PL Pr</td>
</tr>
<tr>
<td>Controls</td>
<td>3.0 0.8 72.7 13.9 9.6</td>
<td>9.9 20.0 14.2 31.3 24.6</td>
<td>16.3 19.2 2.1 24.1 38.3</td>
<td>0.6 2.1 2.5 3.8 91.0</td>
</tr>
<tr>
<td>Cholesterol diet</td>
<td>8.1 39.2 26.6 11.5 14.7</td>
<td>13.4 40.3 1.8 21.5 22.9</td>
<td>18.1 21.9 3.3 30.8 25.9</td>
<td>5.0 16.6 1.4 6.7 70.3</td>
</tr>
</tbody>
</table>

Values are the mean of determinations from two separate lipoprotein preparations.

VLDL, very low density lipoprotein; LDL, low density lipoprotein; HDL, high density lipoprotein; Chol, cholesterol; EC, esterified cholesterol; TG, triglycerides; PL, phospholipid; Pr, protein.

Thus, uptake of \( \beta \)-VLDL and acetylated LDL by mouse peritoneal or human circulating monocytes/macrophages increases ACAT activity by several times, leading to cholesteryl ester accumulation, whereas native LDL uptake does not.\(^{30}\) From the cytoplasmic deposits, cholesteryl esters may be mobilized and exit the cell only after hydrolysis by a cytosolic NCEH. The presence in the cellular environment of suitable cholesterol acceptors is also necessary.\(^{31}\) Accumulation of cholesterol in monocytes/macrophages is thus regulated both by the extracellular concentration of certain species of cholesteryl ester-rich lipoproteins as well as by that of cholesterol acceptors—among others, HDL.\(^{11}\)

Although the events leading to cholesterol accumulation in macrophages have been extensively investigated in vitro, much is still to be understood of the phenomenon in vivo. Feo et al\(^{13}\) measured a twofold to threefold increase in total cholesterol content of peritoneal macrophages in rats treated for 11-13 weeks with a cholesterol-enriched diet, but the time course of the increase was not investigated. These authors found that the diet-induced hypercholesterolemia decreased macrophage phagocytic ability. In the present study we have investigated cholester
The cholesterol accumulation that occurs in peritoneal macrophages is likely generated during peritoneal fluid lipoprotein migration. Given the persistence of these cells in the circulation, we believe it is unlikely that cholesterol accumulation is mediated by peritoneal fluid lipoprotein, given the fact that very limited amounts of cholesterol were carried by them even during the peak of plasma cholesterol concentration. In vitro, β-VLDLs are known to cause macrophage cholesteryl ester accumulation and also to activate ACAT. β-VLDLs are known to circulate in the plasma of cholesterol-fed animals. Analysis of the chemical composition of the plasmatic VLDL from the rats after 1 week of cholesterol feeding is consistent with this idea. Cholesterol esterification activity was also found to be increased in peritoneal monocytes/macrophages at both 3 and 7 days after cholesterol feeding in our experiments. It is thus tempting to speculate that the β-VLDL found in plasma after cholesterol feeding has been responsible for the ACAT activation and the cholesteryl ester accumulation in peritoneal macrophages. Activity of ACAT as well as of NCEH remained steady during dietary manipulation.

Lipoproteins found in peritoneal fluid had compositions very dissimilar from those of any circulating major lipoprotein class. Peritoneal lipoproteins from both animals fed cholesterol or normal rat chow diets had pre-β mobility on agarose gel electrophoresis and appeared to be a homogeneous class of particles of molecular size smaller than that of circulating HDL. The apolipoprotein moiety is composed of apo A-IV, apo E, and apo A-I, the latter being the most abundant protein present. Apo A-I lipid particles with pre-β mobility have been recently identified in plasma by Fielding and play an important role as circulating acceptors of cellular cholesterol. Because of their small size, they may easily invade peripheral tissues. Sloop et al have found that peripheral lymph lipoprotein—mostly apo A-I—lipid complexes—increase their cholesterol content after cholesterol feeding in animals. These authors have concluded that the enrichment is due to uptake of tissue cholesterol, as lymphatic lipoproteins are involved in the early phases of reverse cholesterol transport. Unfortunately however, there is no study in which native lymphatic lipoproteins have been incubated directly with cells to assess their cholesterol efflux potential. It is thus only speculative that we propose that cholesterol enrichment in peritoneal lipoproteins after cholesterol feeding reflects cholesterol efflux from macrophages and perhaps also efflux from other tissues. Further studies will be needed to better investigate this point.

In conclusion, these results support the usefulness of the rat peritoneal macrophage model for studying in vivo cellular changes in cholesterol homeostasis. We observed that within a few days after consumption of a cholesterol-enriched diet, there is significant cholesteryl ester accumulation in peritoneal macrophages. This increase is concomitant with a significant elevation of these cells' ACAT activity. Reversion to a normal diet is accompanied by intracellular cholesterol efflux, with a rapid return to pretreatment levels. These data provide evidence that the plasma lipid level is a major determinant of tissue intracellular cholesterol content and composition.
In Vivo Accumulation of Cholesterol in Macrophages


**KEY WORDS** • monocytes • macrophages • cholesteryl esters • rats
Peritoneal macrophage cholesteryl ester content as a function of plasma cholesterol in rats.
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