Species Difference in Cholesteryl Ester Cycle and HDL-Induced Cholesterol Efflux From Macrophage Foam Cells

Hideki Hakamata, Akira Miyazaki, Masakazu Sakai, Yoshiko Suginoehara, Yu-Ichiro Sakamoto, Seikoh Horiuchi

Abstract  The species difference in the turnover rates of the cholesteryl ester (CE) cycle in macrophage foam cells (MFC) was examined in mice and rats. MFC were induced by acetylated-LDL and pulsed with [H]oleate, followed by a chase with [14C]oleate. The replacement of the initial amount of cholesteryl [H]oleate by cholesteryl [14C]oleate within 24 hours was 63% in mouse MFC, whereas it was 33% in rat MFC. The corresponding replacement in rabbit MFC was < 10%. In addition, HDL removed 41% of the CE mass from mouse MFC but only 22% from rat MFC. HDL-induced CE reduction from mouse MFC was enhanced by 40% with the inhibitor for acyl-coenzyme A:cholesterol acyltransferase (58-035), whereas the enhancing effect was not observed with rat MFC. These results indicate that the rate of CE turnover may serve as a critical factor to determine the capacity of MFC to respond to HDL-induced CE reduction, suggesting the possibility that the species difference in the turnover rates of the CE cycle in MFC might explain, in part, the species difference in susceptibility to experimental atherosclerosis. (Arterioscler Thromb. 1994;14:1860-1865.)

Key Words  • cholesteryl ester cycle  • cholesterol efflux  • HDL  • macrophage foam cells

Macrophage foam cells (MFC) play an essential role in progression of the early stage of atherosclerosis.1 Chemically modified LDLs such as acetylated-LDL and oxidized LDL are recognized by the macrophage scavenger receptors2,3 and undergo receptor-mediated endocytosis, followed by delivery through endosomes to lysosomes.4 In lysosomes, the protein moiety of these modified LDLs is subjected to proteolytic hydrolysis, whereas their cholesterol moiety, mainly cholesteryl esters (CE), undergoes hydrolytic conversion to free (unesterified) cholesterol and free fatty acids by acid lipase, a lysosomal cholesterol esterase.5 Free cholesterol thus generated in lysosomes is then transported across lysosomal membranes into cytoplasm6 and is further delivered to the endoplasmic reticulum, where free cholesterol is reesterified to CE by acyl-coenzyme A:cholesterol acyltransferase (ACAT), leading to CE accumulation in cytoplasm as lipid droplets. In a morphological sense, CE accumulated in these lipid droplets appear to be metabolically inert. In a biochemical sense, however, cytoplasmic CE undergoes a continual shuttle between hydrolysis to free cholesterol by neutral cholesterol esterase and reesterification to CE by ACAT. This CE turnover was first discovered by Brown et al7 in 1980 and was called the “CE cycle.” The presence of the CE cycle in various types of cells has since been established.8-10

We recently demonstrated that in foam cells obtained by incubating rat peritoneal macrophages with acetylated-LDL, the cellular CE level was reduced to 50% to 60% of the initial level after 24-hour incubation with HDL.11,12 However, this rate of HDL-induced CE reduction from rat MFC seemed much slower than that observed in mouse MFC, in which the CE level was reduced to 20% within 24 hours after exposure to HDL.7,13 Moreover, in contrast to mouse MFC, it was reported that the HDL-induced CE reduction was hardly observed in rabbit MFC.14 Because the hydrolytic conversion of CE to free cholesterol was a prerequisite step for HDL-induced cholesterol efflux from plasma membranes, we reasoned that the species difference in the rates of CE reduction from MFC might be due to the difference in the turnover rates of the CE cycle. In the present study, we determined the rates of CE turnover of rat MFC by pulse-chase experiments (pulse with [H]oleate and chase with [14C]oleate) and compared them with those of mouse and rabbit MFC. The results revealed that the rate of CE turnover in mouse MFC was faster than that in rat MFC. The corresponding rate in rabbit MFC was much slower than in the other two species. In addition, the comparison of these rates of CE turnover in MFC with HDL-induced CE reduction from these cells strongly suggests a functional link of the rate of CE turnover not only to the sensitivity of MFC toward HDL-induced CE reduction but also to the species difference in susceptibility to experimental atherosclerosis.

Methods

[14C]Oleic acid (2.07 GBq/mmol) and [9,10(n)-2H]oleic acid (370 GBq/mmol) were purchased from Amersham. Tissue culture media and reagents were obtained from Gibco Laboratories. Silica gel on aluminum sheets for thin-layer chromatography (TLC) was obtained from Merck. Cholesterol ox-

Materials

Received June 21, 1994; revision accepted August 28, 1994.
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dase from Nocardia was from Boehringer. An ACAT inhibitor (compound 58-035) was a generous gift from Dr J.G. Heider of Sandoz, Inc.

**Lipoproteins**

Human LDL (d = 1.019 to 1.063 g/mL) and HDL (d = 1.063 to 1.21 g/mL) were isolated by sequential ultracentrifugation, and traces of apolipoprotein B and E were removed from HDL by a heparin-agarose column. Acetyl-LDL was prepared as described.

**Animals and Cells**

Mouse and rat peritoneal macrophages were collected from nonstimulated male DDDY mice (weight, 25 to 30 g) and male Wistar rats (weight, 170 to 200 g), suspended at 2 x 10⁶/mL with Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal calf serum (FCS), HEPES (10 mmol/L, pH 7.4), streptomycin (0.1 mg/mL), and penicillin (100 U/mL) (medium A).

Rabbit peritoneal macrophages were obtained 4 days after intraperitoneal injection of 100 mL of phosphate-buffered saline (PBS) containing 2 g of soluble starch into each male New Zealand White rabbit (weight, 2 to 2.5 kg). Cells were suspended at 1 x 10⁶/mL in DMEM containing 2% lactic-bumin, HEPES (10 mmol/L, pH 7.4), streptomycin (0.1 mg/mL), and penicillin (100 U/mL) (medium A). To each 35-mm dish was added 1.0 mL of the cell suspension, and the dishes were incubated for 2 hours. Cell monolayers formed were washed three times with 1 mL of PBS and used for the following experiments. More than 95% of the adherent cells were judged to be macrophages by both Giemsa staining and carbon-particle uptake.

**Cellular Assays**

[³H]Oleate and [¹⁴C]Oleate were conjugated with bovine serum albumin (BSA) as described previously. Adhered macrophages were first converted to foam cells by incubation for 12 hours with medium A (rat and mouse) or medium B (rabbit) containing 50 μg/mL of acetyl-LDL. After equilibration for 6 hours in medium C containing 0.1 mmol/L of [³H]Oleate, cells were further incubated with 0.1 mmol/L of [¹⁴C]Oleate in medium C. At the indicated times, cells were harvested for determination of radioactivity of cholesterol [³H]Oleate (a) and cholesteryl [¹⁴C]Oleate (b) as described under "Methods." The symbol m indicates the total contents of radioactive cholesteryl oleate ([³H]+[¹⁴C]). The results are expressed as percentages of the initial amounts of cholesteryl [³H]Oleate. The 100% values were 30.4 nmol/mg cell protein in rat MFC and 34.6 nmol/mg cell protein in mouse MFC. Data are representative of three separate experiments with triplicate wells. Bars represent SD. When the error bars are not shown, they are within the symbols.

**Statistical Analysis**

Data were evaluated by Student's t test. The difference was judged to be significant at P < 0.05.

**Results**

**CE Cycle in Rat and Mouse MFC**

Macrophages were incubated with acetyl-LDL in the presence of [³H]Oleate for labeling of cellular CE and subsequently chased with [¹⁴C]Oleate. The extents of accumulation of cholesteryl [³H]Oleate were almost the same between rat and mouse (Fig 1). In both rat and mouse MFC, cholesteryl [³H]Oleate decreased with time, which was balanced by a compensatory increase in cholesteryl [¹⁴C]Oleate. Total radioactive cholesteryl oleate ([³H]+[¹⁴C]) remained constant. However, the rate of replacement of cholesteryl [³H]Oleate by cholesteryl [¹⁴C]Oleate was different between rat and mouse. The replacement of the initial amount of cholesteryl [³H]Oleate by cholesteryl [¹⁴C]Oleate within 24 hours was 63% in mouse MFC, whereas it was 33% in rat MFC. These results indicated that the rate of CE turnover in mouse MFC was faster than that in rat MFC.

**Effect of HDL on Radioactivity of Cellular Cholesteryl Oleate in Rat and Mouse MFC**

Under conditions identical to those shown in Fig 1, MFC were chased in the presence of 0.25 mg/mL of HDL (Fig 2). The rate of a decrease in cholesteryl [³H]Oleate was not significantly altered by HDL. In contrast, formation of cholesteryl [¹⁴C]Oleate was inhibited to the basal level by the presence of HDL in both rat and mouse MFC, leading to a decrease in the total radioactive cholesteryl oleate. The rate of reduction in total CEs from rat MFC was much slower than that in mouse MFC. In rat MFC, the total radioactive cholesteryl oleate was reduced by 43% over 24 hours (Fig 2, left), whereas it was reduced by 79% in mouse MFC (Fig 2, right). Thus, the reducing effect of HDL on cellular CE was more prominent in mouse MFC than in rat MFC. Taken together with the result obtained in Fig 1, this suggests that CE reduction from MFC would largely depend on the turnover rate of the CE cycle.

**Effects of HDL on Cellular Cholesterol Mass in Rat and Mouse MFC**

The tracer experiment in Fig 2 demonstrated that the rate of HDL-induced CE reduction in mouse MFC was faster than that in rat MFC. This was further examined by determination of cellular cholesterol mass. In the
The initial values of HDL. At the indicated times, cells were harvested for determination of cellular cholesteryl esters (CE) (A) and total cholesterol (B) as described under "Methods." The results are expressed as percentages of the initial amount of cholesteryl [3H]oleate. The 100% values were 30.4 nmol/mg cell protein in rat MFC and 34.6 nmol/mg cell protein in mouse MFC, respectively. Data are representative of two separate experiments with triplicate wells. Bars represent SD. When the error bars are not shown, they are within the symbols.

CE Cycle in Rabbit MFC

It was reported that HDL had little, if any, capacity to reduce cellular CE levels from rabbit MFC. To test whether this might be due to the slow rate of CE turnover, we determined the turnover rate of the CE cycle in rabbit MFC. Rabbit macrophages were similarly converted to foam cells with acetyl-LDL in the presence of [3H]oleate and chased with [14C]oleate. As shown in Fig 4 (left), a change in cholesteryl [3H]oleate and cholesteryl [14C]oleate over 36 hours did not exceed 10% of the initial radioactive activity of cholesteryl [3H]oleate, indicating that the rate of CE turnover in rabbit MFC was much slower than those in rat and mouse MFC. When HDL was added, the formation of cholesteryl [14C]oleate was suppressed to the basal level (Fig 4, right). However, because the rate of CE turnover in rabbit MFC was extremely slow, the capacity of HDL to reduce total cholesteryl oleate ([3H]+[14C]) was negligibly weak (Fig 4, right).

Effect of the ACAT Inhibitor (58-035) on HDL-Induced CE Reduction From Rat and Mouse MFC

We examined the effects of the ACAT inhibitor (58-035) on cholesterol metabolism in rat and mouse MFC. Macrophages were converted to MFC with acetyl-LDL and then exposed to the ACAT inhibitor. When mouse MFC were incubated for 12 hours with 5 µg/mL of the ACAT inhibitor alone, the cellular CE level decreased by 61%, whereas free cholesterol increased by 29% (Table 1). In rat MFC, the CE level decreased by 35%, whereas free cholesterol increased by 10% (Table 2). Thus, it was evident that cellular CE or free cholesterol level in mouse MFC was much more changeable than that in rat MFC, indicating that the turnover rate of the CE cycle in mouse was faster than that in rat.

Next we examined the effect of the ACAT inhibitor on HDL-induced cholesterol efflux from rat and mouse MFC. In mouse MFC, the CE level was reduced by 41% by 12-hour incubation with 0.25 mg/mL of HDL (Table 1). When the ACAT inhibitor was coincubated with
HDL, CE reduction was enhanced from 41% to 56%. Reduction in total cholesterol was also enhanced by the ACAT inhibitor from 30% to 46%, indicating that HDL-induced cholesterol efflux from mouse MFC was enhanced by the ACAT inhibitor. In rat MFC, the CE level was reduced by 22% by the presence of HDL (Table 2). However, in contrast to mouse MFC, the addition of the ACAT inhibitor to rat MFC did not enhance HDL-induced reduction in cellular CE or in total cholesterol (Table 2). Thus, under the present in vitro conditions, the enhancing effect of the ACAT inhibitor on HDL-induced cholesterol efflux from MFC was evident in mouse but not in rat, suggesting that the susceptibility to the ACAT inhibitor would be closely related to the CE cycle of each species.

Discussion

The present study has demonstrated a marked difference among species in the turnover rate of the CE cycle in MFC. The replacement of the initial amount of cholesteryl [1H]oleate by cholesteryl [14C]oleate within 24 hours was 63% in mouse MFC, whereas it was 33% in rat MFC (Fig 1). The corresponding replacement in rabbit MFC was <10% (Fig 4). Moreover, comparison of these rates of CE turnover with those of HDL-induced CE reduction from MFC provided an interesting result. Because the hydrolytic conversion of CE to free cholesterol was a rate-limiting step for HDL-induced CE reduction, the rate of cholesterol efflux from MFC of each species was largely dependent on the rate of CE turnover (Figs 2 and 3). Although previous studies already implicated the presence of the species difference in the rates of HDL-induced CE reduction from MFC,14,23 the present study made it clear for the first time that the turnover rate of the CE cycle in each species was one of the major factors determining the rates of HDL-induced CE reduction from MFC.

Although mouse macrophages have frequently been employed for the experiments of cholesterol efflux from MFC,7,13,24,25 we previously used rat macrophages because peritoneal macrophages obtained from a nonstimulated rat were severalfold higher in number than those obtained from a mouse.11,12 Using rat MFC, we demonstrated that HDL could decrease cellular CE by 40% to 50% within 24 hours.11,12 However, this rate of CE reduction seemed slower than those previously reported with mouse MFC.7,13 Moreover, a morphological study demonstrated that cytoplasmic lipid droplets of foam cells in aortic explants from cholesterol-fed rabbits contained CE even after they were exposed to HDL, whereas CE droplets in mouse MFC disappeared rapidly by a similar treatment with HDL.26 In support of this notion, an in vitro study with rabbit MFC did not show any appreciable effect of HDL on cellular CE levels.14 These observations suggested that the cholesterol metabolism in macrophages, and their sensitivity to HDL in particular, would vary among these species.

Previous reports demonstrated that the ACAT inhibitor (58-035) could enhance HDL-induced cholesterol efflux from mouse MFC.24,25 This phenomenon was also observed in the present study (Table 1). However, in contrast to mouse MFC, the ACAT inhibitor had little, if any, effect on HDL-induced cholesterol efflux from rat MFC (Table 2). In general, ACAT inhibitors are believed to be able to enhance HDL-induced cholesterol efflux because it increases the cellular free cholesterol pool accessible to HDL.25 We observed in the present study that the addition of the ACAT inhibitor alone increased cellular free cholesterol by 30 nmol/mg cell protein in mouse MFC, whereas the corresponding

## Table 1. Effects of HDL and the ACAT Inhibitor (58-035) on Cellular Cholesterol Mass in Mouse Macrophage Foam Cells

<table>
<thead>
<tr>
<th></th>
<th>FC, nmol/mg Cell Protein</th>
<th>CE, nmol/mg Cell Protein</th>
<th>TC, nmol/mg Cell Protein</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>108.4±6.6 (100)</td>
<td>72.4±10.9 (100)</td>
<td>180.8±16.9 (100)</td>
</tr>
<tr>
<td>58-035</td>
<td>139.5±10.7 (128.7)</td>
<td>28.3±6.4 (39.1)</td>
<td>167.9±13.8 (92.9)</td>
</tr>
<tr>
<td>HDL</td>
<td>84.1±0.6 (77.6)*</td>
<td>42.7±7.3 (59.0)†</td>
<td>126.8±6.7 (70.1)‡</td>
</tr>
<tr>
<td>HDL+58-035</td>
<td>66.3±6.3 (61.2)*</td>
<td>31.8±2.0 (43.9)†</td>
<td>98.1±8.0 (54.3)‡</td>
</tr>
</tbody>
</table>

FC indicates free cholesterol; CE, cholesteryl esters; and TC, total cholesterol. Cells were prepared and harvested for determination of cellular cholesterol mass as described under Methods. Values are mean±SD in quadruplicate wells. Percentages of the control values are expressed in parentheses. Statistical significance by Student's t test: *P<.01; †P<.05; ‡P<.01.

## Table 2. Effects of HDL and the ACAT Inhibitor (58-035) on Cellular Cholesterol Mass in Rat Macrophage Foam Cells

<table>
<thead>
<tr>
<th></th>
<th>FC, nmol/mg Cell Protein</th>
<th>CE, nmol/mg Cell Protein</th>
<th>TC, nmol/mg Cell Protein</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>109.8±5.5 (100)</td>
<td>56.5±4.1 (100)</td>
<td>168.0±3.7 (100)</td>
</tr>
<tr>
<td>58-035</td>
<td>120.1±7.2 (109.6)</td>
<td>38.2±9.5 (65.3)</td>
<td>158.1±14.3 (94.1)</td>
</tr>
<tr>
<td>HDL</td>
<td>81.9±3.3 (74.7)*</td>
<td>45.5±6.3 (77.8)†</td>
<td>127.4±9.4 (75.8)‡</td>
</tr>
<tr>
<td>HDL+58-035</td>
<td>86.5±5.2 (78.9)*</td>
<td>42.7±3.4 (73.0)†</td>
<td>129.9±8.2 (77.3)‡</td>
</tr>
</tbody>
</table>

FC indicates free cholesterol; CE, cholesteryl esters; and TC, total cholesterol. Cells were prepared and harvested for determination of cellular cholesterol mass as described under Methods. Values are mean±SD in quadruplicate wells. Percentages of the control values are expressed in parentheses. Statistical significance by Student's t test: *††not significant.
increase in free cholesterol in rat MFC was <10 nmol/mg cell protein, probably because of the slower turnover rate of CE (Tables 1 and 2). It is likely, therefore, that the enhancing effect of the ACAT inhibitor on HDL-induced cholesterol efflux would be closely correlated with the rate of CE turnover. In a related issue, the morphological study by Robenek and Schmitz disclosed that an ACAT inhibitor, oomticate, could induce the formation in mouse MFC of lamellar bodies that were composed of free cholesterol and phospholipids originating from cytoplasmic lipid droplets. These lamellar bodies first fused with endosomes containing HDL particles and then disappeared during the retroendocytosis and subsequent resecretion of HDL to the extracellular medium.

Because the turnover rate of the CE cycle is determined by both ACAT and neutral cholesterol esterase, it is important to know the factors regulating these enzymes and their molecular mechanism. Neutral cholesterol esterase of mouse macrophage cell lines such as J774 and P388D1 was stimulated by cyclic AMP. Although the molecular structure of neutral cholesterol esterase has not been clarified, its enzyme activity in a mouse macrophage cell line (WEHI) was completely blocked by the antibody specific for hormone-sensitive lipase, an enzyme that catalyzes triglyceride hydrolysis, indicating that neutral cholesterol esterase would be identical or closely related to hormone-sensitive lipase. A regulatory mechanism of ACAT activity is more complicated to understand at present. Using the microsomal (nonreconstituted) ACAT assay in which free cholesterol of microsomal fractions was used as a substrate, previous studies showed that the ACAT activity was increased or induced by cholesterol loading. However, by using the reconstituted assay in which free cholesterol content of microsomal fractions was enriched with exogenous cholesterol and used as a cholesterol substrate, Cadigan and Change showed that there was no significant difference in the ACAT activity between cholesterol-loaded and -nonloaded cells. It seems likely, therefore, that the increase in the ACAT activity of cholesterol-loaded cells determined by microsomal assay does not simply imply the increase in the enzyme activity itself but rather reflects an increase in cholesterol contents in microsomal fractions. Recent success in cDNA cloning of human ACAT would help greatly in solving this issue at a molecular level.

It is generally accepted that mice and rats are much more resistant to diet-induced atherosclerosis than rabbits. Although the susceptibility to atherosclerosis would be multifactorial, it could be explained, in part, by the species difference in the turnover rates of the CE cycle in MFC.

Acknowledgments

This work was supported by a Grant-in-Aid for Scientific Research (06770110) from the Ministry of Education, Science, and Culture of Japan and a grant from the HMG-CoA Reductase Research Foundation. We are grateful to Tomohiro Nishi for his excellent technical assistance during this study.

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Species difference in cholesteryl ester cycle and HDL-induced cholesterol efflux from macrophage foam cells.

H Hakamata, A Miyazaki, M Sakai, Y Suginoohara, Y Sakamoto and S Horiuchi

Arterioscler Thromb Vasc Biol. 1994;14:1860-1865
doi: 10.1161/01.ATV.14.11.1860

Arteriosclerosis, Thrombosis, and Vascular Biology is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 1079-5642. Online ISSN: 1524-4636

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