Genes of Cholesterol Metabolism in Human Atheroma
Overexpression of Perilipin and Genes Promoting Cholesterol Storage and Repression of ABCA1 Expression

Fabien Forcheron, Liliana Legedz, Guiletta Chinetti, Patrick Feugier, Dominique Letexier, Giampierro Bricca, Michel Beylot

Objective—Accumulation of cholesterol in foam cells of atheroma plaques depends on the balance between uptake and efflux of cholesterol. It may also depend on proteins surrounding lipid droplets, adipophilin, and perilipins. They favor triglyceride storage in adipocytes and could play a similar role for cholesterol in atheroma.

Methods and Results—We measured in human atheroma and nearby macroscopically intact tissue (MIT) the expression of perilipin, adipophilin, and regulatory factors of cholesterol metabolism. We identified perilipin A in human arterial wall. Its expression was largely increased in atheroma compared with MIT, and perilipin was present in macrophages and vascular smooth muscle cells. Adipophilin, ACAT1, and CD36 were also overexpressed in atheroma. mRNA levels of low-density lipoprotein receptor, 3-hydroxy-3-methylglutaryl coenzyme A reductase, and SREBP-2 were unchanged. With respect to efflux of cholesterol, the mRNA levels of NCEH and ABCA-1 were unchanged, whereas CLA-1 mRNA was slightly higher in atheroma. Importantly, immunoblotting of ABCA-1 showed a dramatic decrease of ABCA1 protein, the key molecule of cholesterol efflux, in atheroma compared with MIT.

Conclusion—We show the presence and induction of perilipin in atheroma. This overexpression and the coordinated modifications of expression of key regulatory factors for cholesterol metabolism could favor cholesterol accumulation.


Key Words: atherosclerosis • genes • lipids • macrophages • smooth muscle cells

Accumulation of cholesterol esters (CEs) in macrophages and smooth muscular cells (SMCs) transforming them in foam cells is a hallmark of atherosclerosis.1 This accumulation depends on the balance between the uptake of cholesterol-rich lipoproteins such as oxidized low-density lipoprotein (LDL)2 and the efflux of free cholesterol (FC) controlled by the transporter ABCA13 and to a less extent by CLA-1 (or SR-B1).4,5 CE accumulation depends also on the intracellular metabolism of cholesterol. CE taken up by cells is hydrolyzed in lysosomes to FC that is directed to various cell membranes by the protein NPC-1 (Nieman-Pick C1 protein).6 FC can also be produced by endogenous synthesis. Because excessive accumulation of FC is toxic for cells,7 FC must be either removed through efflux to extracellular acceptors such as high-density lipoprotein or esterified. This esterification depends in macrophages on the microsomal enzyme acyl-CoA cholesterol acyl-transferase 1 (ACAT-1)8 and on the availability of long-chain fatty acyl CoA.9 CE stored in lipid droplets can be removed from cells only after hydrolysis to FC by a cholesterol ester hydrolase, whose nature is still debated. In murine macrophages, CE seems to be hydrolyzed by hormone-sensitive lipase (HSL), the enzyme controlling the hydrolysis of triglycerides (TGs) stored in adipocytes.9 However, HSL is not expressed in human macrophages10 and CE is probably hydrolyzed in these cells by the neutral cholesterol ester hydrolase (NCEH) cloned by Ghosh,11 also called triacylglycerol hydrolase and carboxylesterase 3.12

Storage of lipids in droplets is also dependent in cells accumulating TG (mainly adipocytes and hepatocytes) or CE (steroidaligens cells) on specific proteins surrounding these droplets, particularly adipophilin (or ADRP) and the perilipins.13,14 ADRP is considered to have a role in the transport of lipids from cell membranes to lipid droplets and is present in all cells storing lipids.14 ADRP is expressed in foam cells of atherosclerotic plaques.15 Its expression increases during differentiation of monocytes into macrophages and incubation of macrophages with oxidized LDL.15,16 Its overexpression in THP-1 macrophages enhances lipid accumulation.17 There are 3 different perilipins, A, B, and C, resulting from alternative splicing of a common premessenger RNA.18 Perilipins A and B are found in adipocytes, with the A form being predominant. Perilipin C is found in steroidogenic cells. In adipocytes, in the basal state, ie, in the absence of

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stabilization of lipolysis, perilipin protects TG from hydrolysis by HSL. When lipolysis is stimulated by β-adrenergic agents, perilipins are phosphorylated on specific serine sites and allow phosphorylated HSL to hydrolyze TG. However, this role of perilipin in the control of lipid storage has been demonstrated only for perilipin A and in adipocytes. The expression of perilipins was considered to be limited to adipocytes and steroidogenic cells. Recently, evidence for the presence of perilipin in atheroma plaques has been presented. Perilipin could therefore be implicated in the development of atherosclerosis by controlling as in adipocytes the hydrolysis of stored lipids. However, the nature (A, B, or C) of the perilipin expressed in atheroma plaques was not determined, an important point because the role of perilipin in the control of lipolysis in adipocytes has been demonstrated only for perilipin A.

We determined in the present report the expression level of perilipin, ADRP, and main genes controlling cholesterol metabolism in human atherosclerotic plaques compared with nearby macroscopically intact tissue (MIT). We show that perilipin is overexpressed, as ADRP, in human atheroma. The perilipin form is perilipin A and it is expressed in macrophages and SMCs. Lastly, these modifications of ADRP and perilipin expression in atheroma appear associated with coordinated modifications of the expression of other genes controlling cholesterol metabolism in a way promoting the synthesis and storage of CE.

Methods

Human Subjects

The procedure was approved by local ethical committee. Human atheroma plaques were removed during carotid endarterectomy from 10 subjects (5 males, 5 females, aged 68±3 years) who gave their informed consent. Five subjects had overt type 2 diabetes. The others had evidence of insulin resistance with moderate increase in basal level of plasma glucose and/or insulin concentrations. Four subjects (among whom 3 had type 2 diabetes) were treated with statins. Samples collected directly in the surgery room were divided into 2 mates (among whom 3 had type 2 diabetes) were treated with statins. Samples collected directly in the surgery room were divided in 2 parts. Atherosclerotic plaque and MIT situated in the vicinity of the plaque, and were frozen in liquid nitrogen (LN₂) or fixed for immunohistology. A fragment of MIT was spared for SMC culture.

Studies in Rats

Male Sprague-Dawley rats, obese Zucker rats, and their lean littermates (Charles River, L’Arbresle, France) were anesthetized with pentobarbital in the fed state. After blood removal for determination of plasma total cholesterol and TG levels (enzymatic methods), aorta was removed, carefully cleaned from adjacent fibrous and adipose tissue, and quickly frozen in LN₂. In lean and obese Zucker rats, half of the aorta was used for culture of vascular SMC.

Cells Culture

Mononuclear cells isolated from blood of healthy donors (Ficoll gradient) were suspended in RPMI 1640 medium containing gentamicin (40 mg/mL), glutamine (0.05%), and 10% pooled human serum. Monocytes differentiation into macrophages occurs spontaneously by adhesion into the culture dishes. After 10 days of culture, mature monocytes-derived macrophages were in a first experiment incubated with AcLDL (100 μg/mL) during 12, 24, 48, and 72 hours. In a second experiment, monocytes-derived macrophages were incubated 5 or 8 days with 100 or 200 μg/mL of AcLDL. THP-1 cells (ATCC, Rockville, Md) were suspended in RPMI 1640 medium containing calf serum (10%), D-glucose (0.45%), 10 mmol/L HEPES, 1 mmol/L sodium pyruvate, penicillin (100 U/mL), streptomycin (100 mg/mL), 2 mmol/L glutamine, and bicarbonates (7.5%). Human SMC were obtained from MIT. Briefly, in the surgery room, MIT was placed in culture medium (Promocell SMC growth medium) and immediately transported to the laboratory. Tissue was minced in culture dishes and cells grown in the same medium. Cells used for measurements were at passage P3 and P4 and maintained 24 hours without serum before mRNA measurements. SMC from rat aortas were obtained after the same general protocol.

mRNA

Purified total RNA samples (Trizol; Gibco-Brl, Cergy Pontoise, France) were quantified by spectrophotometry and their integrity checked by agarose gel electrophoresis. ABCA1 and SREBP-1c mRNA levels were measured by competitive reverse-transcription (RT) polymerase chain reaction (PCR). CD36 mRNA concentrations were determined by RT-PCR using cyclophilin as reference. Other mRNA, LDL receptor (LDL-R), 3-hydroxy-3-methylglutaryl (HMG)-CoA reductase, SREBP-2, NPC1, ACAT1, NCEH, CLA-1, LXRα, and stearal-CoA desaturate (SCD) were measured by RT-PCR using β-actin as internal standard. β-actin mRNA levels were measured by competitive RT-PCR with a specific competitor to detect and take in account possible variations of abundance between MIT and atherosclerotic plaques. mRNA concentrations were expressed as ratio versus a normalized amount (100 attomoles/μg of total RNA) of β-actin mRNA level. The presence of some mRNA (HSL, resistin) was checked by using in RT-PCR only primers specific for these targets. Primers are shown in Table 1.

ImmunobLOTS

Samples were homogenized in PBS with proteases inhibitors and protein concentrations measured with the BCA kit (Pierce). Proteins (100 μg) were analyzed by SDS-PAGE and transferred to polyvinylidene fluoride (PVDF) membrane. The membranes were probed with a polyclonal guinea pig anti-human perilipin antibody (Progen, Heidelberg, Germany), a polyclonal rabbit anti-human ABCA1 antibody (Novus Biologicals, Littleton, Colo), or a polyclonal goat anti-human SCD antibody (Santa Cruz Tech), followed with incubation of anti-IgG conjugated with peroxidase (Sigma, St Louis, Mo; 1/1000) and revelation with ECL. The density of the bands was determined by densitometry (Image Quant, Molecular Dynamics, Calif). Samples form the plaque and MIT of each subject were run in the same immunoblot.

Immunohistology

Fixed samples were sectioned (4 μm), deparaffined (xylene), and hydrated. Sections were separated in 2 parts. One was incubated with the polyclonal guinea pig anti-human perilipin antibody (Progen, Heidelberg, Germany), and the monoclonal mouse anti-human α actin antibody (1/50) (DAKO, Glostrup, DK) or the monoclonal mouse anti-human CD 68 antibody (1/50) (Santa Cruz Biotechnology, Santa Cruz, Calif) for 1 night at 4°C and the second served as control for nonspecific binding of secondary antibody. The 2 sections were then incubated with anti-guinea pig IgG conjugated with alexa fluor red (1/200) and with anti-mouse IgG conjugated with fluorescein isothiocyanate (1/200) for 90 minutes. Controls were performed by incubating samples only with secondary antibodies.

Statistics

Results are shown as mean and SEM. Comparisons between MIT and atheroma plaques were performed by Student t test for paired values. Comparisons between diabetic and nondiabetic subjects and between subjects treated or not with statins were performed by Mann-Whitney test.

Results

Perilipin A Is Expressed in Arterial Wall and Is Overexpressed in Atherosclerotic Plaques

Perilipin mRNA was present in MIT and in plaques with concentrations clearly increased in plaques (Figure 1).
Comparable results were observed in samples from diabetic and nondiabetic subjects, as well as from subjects treated or not with statins. Neither resistin nor HSL mRNA could be detected in any sample running out contamination of samples by adipose tissue. Immunoblots using an antibody raised against the common (N-terminal) part of perilipin A, B, and C detected a protein with an apparent molecular weight ≈55 kDa. This protein was also more abundant in plaque than in the MIT of the same subject (Figure 1). The presence of perilipin was confirmed by immunohistology. Again, the staining was more abundant in plaque than in MIT and perilipin was present in macrophages and SMCs loaded with cholesterol as shown by colocalization studies with CD68 (marker of macrophages). Right, Staining for perilipin. Middle, Staining - actin. Left, Merging of the 2 stainings.

**Figure 1.** Left panel, mRNA concentrations of perilipin in atherosclerotic plaques (ATH) and in adjacent macroscopically intact tissue (MIT). *P*<0.05 vs MIT (n=10). Right panel, Representative immunoblot and expression level of perilipin protein in plaques and in MIT. *P*<0.05 vs MIT (n=6).

**Figure 2.** Immunohistological studies of perilipin localization in atheroma. The upper panel shows the colocalization of perilipin and CD68 (marker of macrophages). Right, Staining for perilipin. Middle, Staining for CD68. Left, Merging of the 2 stainings. Lower panel, Colocalization of perilipin and α-actin (marker of SMCs). Right, Staining for perilipin. Middle, Staining for α-actin. Left, Merging of the 2 stainings.
resulting in the presence of a codon stop and the synthesis of Perilipin B results from the lack of splicing of intron 8 primer) and exon 9 (antisense primer). The size of the parts of perilipin mRNA situated within exon 8 (sense form). Therefore, immunoblots were analyzed using an antibody raised against the specific C terminal part of perilipin A (dephosphorylated form) or 65 to 67 kDa (phosphorylated form). However, the apparent molecular weight of adipocytes perilipin A during immunoblot analysis is usually 62 kDa (molecular weight 56.8 kDa) than B (molecular weight 46.2). The apparent molecular weight of the protein detected by immunoblot (55 kDa) was more compatible with perilipin A and of amino acids sequence common to perilipin A and B.

The primers and antibody used recognized part of cDNA and of amino acids sequence common to perilipin A and B. The apparent molecular weight of the protein detected by immunoblot (55 kDa) was more compatible with perilipin A (molecular weight 56.8 kDa) than B (molecular weight 46.2). However, the apparent molecular weight of adipocytes perilipin A during immunoblot analysis is usually 62 kDa (dephosphorylated form) or 65 to 67 kDa (phosphorylated form). Therefore, immunoblots were analyzed using an antibody raised against the specific C terminal part of perilipin A (Affinity Bioreagents, Golden, Colo). This antibody recognized the same protein of apparent molecular weight of 55 kDa. We also performed RT-PCR using primers recognizing parts of perilipin mRNA situated within exon 8 (sense primer) and exon 9 (antisense primer). The size of the fragment amplified with the mRNA of perilipin A is 367 bp. Perilipin B results from the lack of splicing of intron 8 resulting in the presence of a codon stop and the synthesis of a protein lacking amino acids encoded by exon 9. If intron 8 is present in perilipin mRNA, the use of primers recognizing sequences situated in exon 8 and 9 will result in the amplification by PCR of a fragment of 1360 bp. We observed only a fragment of 367 bp. Lastly, we performed RT-PCR using sense and anti-sense primers recognizing sequences situated, respectively, in exon 8 and intron 8 giving, if mRNA containing intron 8 of perilipin is present, a fragment of 368 bp. No such fragment was obtained. Therefore, the perilipin expressed in cells of human arterial wall is perilipin A.

Immunohistology of atherosclerotic plaques showed that perilipin was present in macrophages and SMCs. We checked for perilipin in human mononuclear cells, in cultured THP-1 cells, and in human macrophages differentiated from circulating monocytes and studied before and during incubation with acetylated LDL. ADRP (mRNA) was expressed in all these cells and this expression was increased by cholesterol loading as expected (data not shown). However, perilipin mRNA was undetectable, except at very low level, in some human macrophages incubated with Ac-LDL. On the contrary, perilipin (mRNA) was expressed in cultured SMC of human atherosclerotic carotid artery (Figure 3) and, also, to a lower level, in cultured SMC from aorta of obese Zucker rats. ADRP was also clearly expressed in these cells.

Expression of Other Genes of Cholesterol Metabolism

NPC1, ADRP, and ACAT1 were expressed in MIT and this expression was increased inside the plaques (Table 2) for ADRP and ACAT1. NCEH was clearly expressed in MIT and atheroma without difference in mRNA levels between these 2 tissues. On the contrary, we could not detect any HSL mRNA in diabetic and nondiabetic subjects and in patients treated or not with statins.

mRNA concentrations of HMG-CoA reductase and LDL-r were slightly but not significantly increased in plaques, whereas those of CD36 was markedly increased in plaques (P<0.05; Table 2). The increase in CD36 mRNA level in plaques was observed irrespective of the presence or not of type 2 diabetes or of treatment with statins. mRNA concentrations of SREBP-2 were identical in MIT and in plaques. These results suggest that pathways for cholesterol uptake and synthesis are not repressed in cells of atheroma plaques and may even be overexpressed (CD36).

CLA-1 mRNA levels were moderately increased (Table 2). ABCA1 mRNA levels were unchanged in plaques despite an increase of mRNA levels of LXRα, a transcription factor stimulating ABCA1 expression. Importantly, contrasting with the lack of variation of mRNA concentration, we observed in immunoblots a dramatic decrease in atheroma plaques of the amount of the protein ABCA1 (Figure 4). We checked whether the expression of other targets of LXRα was increased. Srebp-1c mRNA concentrations were very low, and not different, in plaques and MIT. SCD mRNA concentrations were increased in the plaques (0.33±0.06 versus 0.20±0.04 in MIT; P<0.05, n=6). However, when performing immunoblot, the amount of SCD protein was low in MIT and in plaques without detectable difference between the 2 tissues.

<table>
<thead>
<tr>
<th>mRNA Concentrations Expressed Either as attomoles/μg of Total RNA (ABCA1 mRNA) or Relative to β-actin mRNA (All Other mRNA)</th>
<th>MIT</th>
<th>ATH</th>
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</thead>
<tbody>
<tr>
<td>ADRP</td>
<td>1.07±0.34</td>
<td>2.39±0.71*</td>
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<tr>
<td>ACAT1</td>
<td>0.68±0.38</td>
<td>1.16±0.42*</td>
</tr>
<tr>
<td>NCEH</td>
<td>0.65±0.29</td>
<td>0.80±0.26</td>
</tr>
<tr>
<td>NPC-1</td>
<td>1.58±0.84</td>
<td>2.14±0.91</td>
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<tr>
<td>LDL-r</td>
<td>0.50±0.32</td>
<td>0.70±0.27</td>
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<tr>
<td>HMG-CoA reductase</td>
<td>0.76±0.52</td>
<td>0.92±0.48</td>
</tr>
<tr>
<td>SREBP-2</td>
<td>0.88±0.71</td>
<td>0.84±0.65</td>
</tr>
<tr>
<td>CD36</td>
<td>1.10±0.38</td>
<td>9.70±2.40*</td>
</tr>
<tr>
<td>CLA-1</td>
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<td>1.81±0.41</td>
</tr>
<tr>
<td>LXRα</td>
<td>0.88±0.30</td>
<td>1.43±0.29*</td>
</tr>
<tr>
<td>ABCA1</td>
<td>5.60±2.40</td>
<td>5.09±1.50</td>
</tr>
</tbody>
</table>

* P<0.05 vs the corresponding mRNA value in MIT.
N=10 except for NPC-1 (n=6).

**Figure 3.** Left, Perilipin mRNA is present in human vascular SMCs (hVSCMs) from atherosclerotic artery and in aorta from Sprague-Dawley rats (SD rats). WAT, perilipin mRNA from white adipose tissue. Right, Immunoblot of perilipin for adipose tissue samples (WAT) and aorta of Sprague-Dawley rats.
The present report clearly shows that perilipin is expressed, at the mRNA and protein level, in human arterial wall in the vicinity of atherosclerotic plaques and that this expression is increased inside the plaques. This presence of perilipin is not caused by a contamination by adipose tissue because specific markers of adipose tissue were not detected in any sample. Therefore, the expression of perilipin in arterial wall appears more large than reported by Faber et al, who found perilipin in unstable, ruptured, atheroma plaques. Perilipin could also be expressed in human arterial wall in the absence of atherosclerosis because both its mRNA and protein were found in normal arterial wall of control rats. Immunohistology of human carotid endarterectomy pieces showed that perilipin was present in foam cells derived from both macrophages and SMC. We found also perilipin mRNA in cultured SMC originating from human atherosclerotic carotid artery and from the aorta of obese hyperlipidemic Zucker rats. Perilipin protein has recently been found in THP-1 macrophages and from the aorta of obese hyperlipidemic Zucker rats. This action of fatty acids could be mediated through the nuclear receptors PPARγ because it has been shown that the promoter of the perilipin gene contains functional PPAR-responsive elements and that PPARγ agonists stimulate in adipocytes the expression of perilipin.

Perilipin opposes in adipocytes in the basal state the hydrolysis of TGs by HSL. On stimulation of lipolysis by β-adrenergic agonists, perilipin is phosphorylated by the protein kinase PKA and allows phosphorylated HSL to access lipid droplets and hydrolyze TGs. This mechanism has been demonstrated for perilipin A but not for perilipin B. We have evidence that the perilipin expressed in arterial wall is indeed perilipin A. Perilipin could thus have in foam cells a role comparable to the one played in adipocytes, ie, protect in the basal state CE stored in lipid droplets from hydrolysis, not by HSL as in adipocytes because HSL was not found expressed in human arterial wall, but by NCEH and allow this hydrolysis after phosphorylation. NCEH activity is stimulated by dibutyryl cAMP in macrophages and by both PKA and PKC in rat aorta. The simultaneous hydrolysis of CE by NCEH and esterification of FC by ACAT1 result in a permanent intracellular cycling between CE and FC. ACAT1 and NCEH mRNA were more abundant in atherosclerotic plaques, but this increase was significant only for ACAT1. If these modifications of mRNA concentrations are also present at the level of enzyme activities, they would favor storage of CE. The overexpression in plaques of perilipin opposing the action of NCEH would further shift the balance of this FC–CE cycle toward cholesterol esterification and storage.

We also observed differences in the expression of other important regulatory genes of cholesterol metabolism in plaques compared with MIT. The mRNA concentrations of CD36 were increased, whereas those of LDL-r, contributing also to the cellular uptake of cholesterol, and of HMG-CoA reductase, the key regulatory enzyme of cholesterol synthesis, were unchanged, suggesting that the pathways for both cholesterol synthesis and uptake were not repressed in foam cells of the plaques compared with MIT. Overexpression of CD36 agrees with previous studies showing that its expression is stimulated in cholesterol-loaded macrophages. The trend for higher expression of LDL-r and HMG-CoA reductase is surprising because the expression of these genes is repressed by cellular accumulation of cholesterol and stimulated when cells are depleted of cholesterol. However, it is also possible that the expression of these genes was already near maximally suppressed in MIT.

Cellular cholesterol efflux is controlled by ABCA1 and, to a lesser degree, by CLA-1 that can promote either cholesterol efflux or uptake. There was in plaques a dramatic decrease in the protein amount of ABCA1, suggesting that cholesterol...
efflux was largely decreased. This decrease in ABCA1 protein was not caused by a diminished transcription because mRNA levels were unchanged. Moreover, mRNA concentrations of LXRα,35,36 the main nuclear factor stimulating the transcription of ABCA1, were increased. ABCA1 is also regulated at the post-translational level via changes in the turnover of ABCA1 protein.37–39 Unsaturated fatty acids37–39 regulate at the post-translational level via changes in the protective role by shifting the balance between FC and CE. Lipin could therefore have on the contrary a favorable and protective effects on cells and could play a key role in the evolution of atheroma plaques toward instability.7,41–43 Perilipin could therefore have on the contrary a favorable and protective role by shifting the balance between FC and CE towards CE.

Acknowledgments

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References

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