Enrichment of Acyl Coenzyme A:Cholesterol O-Acyltransferase Near Trans-Golgi Network and Endocytic Recycling Compartment

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Abstract—Acyl coenzyme A:cholesterol O-acyltransferase (ACAT) is the enzyme responsible for cholesterol esterification in macrophages leading to foam cell formation. The determination of its localization is a critical step in understanding its regulation by cholesterol. Using immunofluorescence and confocal microscopy, we previously showed that the enzyme colocalized with markers of the endoplasmic reticulum, but in addition, ACAT was found in an unidentified paranuclear site. In the present study, we further define the localization of paranuclear ACAT. First, we found that ACAT does not colocalize with sorting endosomes or late endosomes labeled with fluorescent α2-macroglobulin. The paranuclear ACAT is close to the endocytic recycling compartment labeled with fluorescent transferrin. We also show that the paranuclear structure containing ACAT is very close to TGN38, a membrane protein of the trans-Golgi network (TGN), but farther from Gos28, a marker of cis, medial, and trans Golgi. After treatment with nocodazole, the central localization of ACAT did not colocalize with markers of the TGN. These data indicate that a significant fraction of ACAT resides in membranes that may be a subcompartment of the endoplasmic reticulum in proximity to the TGN and the endocytic recycling compartment. Because the TGN and the endocytic recycling compartment are engaged in extensive membrane traffic with the plasma membrane, esterification of cholesterol in these membranes may play an important role in macrophage foam cell formation during atherogenesis. (Arterioscler Thromb Vasc Biol. 2000;20:1769-1776.)

Key Words: cholesterol esterification ■ organelles ■ immunolocalization ■ acyl coenzyme A:cholesterol O-acyltransferase

During atherogenesis, macrophages accumulate large amounts of free cholesterol and cholesterol ester droplets, giving the cell a foamy appearance. These modified macrophages play an important role in the initiation and development of atherogenic lesions. When the cellular content of cholesterol reaches a threshold, the enzyme acyl coenzyme A:cholesterol O-acyltransferase (ACAT), an integral membrane protein, catalyzes the esterification of cholesterol. This esterification is a regulated process, but the regulatory mechanisms remain unclear. It has been shown that atherogenic lipoproteins activate ACAT activity in macrophages and other cells, without significantly increasing the mRNA or protein levels. Several hypotheses have been proposed to explain the stimulation of ACAT. One possibility is that cholesterol itself acts as an allosteric regulator of the enzyme. A second possibility is that cholesterol levels in membranes indirectly activate ACAT by modifying the biophysical properties of the membranes in which the enzyme resides. A third possibility is that increasing the cellular cholesterol levels increases the delivery of cholesterol to ACAT or makes cholesterol more accessible to ACAT. One of the key issues in understanding these mechanisms of regulation is to determine the cellular localization of ACAT in macrophages and its relationship to cholesterol distribution in the cell.

The plasma membrane is the major site of free cholesterol in cells. However, studies using filipin labeling have shown that the trans-Golgi network (TGN) is an important intracellular site of free cholesterol in membranes. Using dehydroergosterol (DHE), a fluorescent analogue of cholesterol, it was shown that the endocytic recycling compartment (ERC) is another major intracellular site of free cholesterol in fibroblasts, and this finding was confirmed by filipin staining. Several studies have shown that the endoplasmic reticulum (ER) bilayer is relatively low in free cholesterol content, even though it is the site of cholesterol synthesis. The pathway of cholesterol delivery to ACAT is not well characterized, but several lines of evidence support the hypothesis that most of the cholesterol that is esterified by ACAT has passed through the...
ER.17, 18 Because very little endocytic membrane traffic in fibroblasts have shown that ACAT is found mostly in the ER, it is difficult to envisage a large flux of cholesterol from the plasma membrane to the ER by conventional membrane trafficking pathways. Specialized cholesterol transport processes have been proposed,20 but the evidence in support of these processes remains mostly indirect at present. Recently, we showed by immunofluorescence and confocal microscopy that although most ACAT colocalized with an ER marker in peritoneal macrophages, a portion of it was concentrated in a paranuclear site that was not enriched for ER markers.15

In the present study, we further characterized this paranuclear localization of ACAT. We found that ACAT was close to the TGN and the ERC, both of which are cholesterol-rich organelles.9 The close apposition of ACAT with these cholesterol-rich organelles may provide a mechanism for localized control of cholesterol content in their membranes, and their cholesterol levels can affect membrane trafficking processes.21,22 Because the TGN and the ERC are engaged in extensive membrane traffic with the plasma membrane, esterification of cholesterol derived from these membranes may play an important role in macrophage foam cell formation during atherogenesis.

Materials

Tissue media and reagents and calf serum were obtained from Life Technologies Inc or GIBCO-BRL. FBS was purchased from Gemini Bioproducts or GIBCO-BRL. Mouse IgG2a and rat antibodies to the mouse CD12/CD32 Fc receptor were from Chemicon and PharMingen, respectively. Rabbit anti-ACAT antibodies16 were generously provided by Dr Robert V. Farese, Jr (Gladdstone Foundation for Cardiovascular Research and University of California, San Francisco), and their specificity in murine macrophages has been documented previously.15 To make guinea pig anti-ACAT antibodies, the amino-terminal 120–amino acid residues of mouse ACAT were expressed as a fusion protein in Escherichia coli with the use of the pGEX-2T expression vector. The fusion protein was purified with glutathione-Sepharose beads (Amersham Pharmacia Biotech) and used to generate antiserum in guinea pigs as described.22 Rabbit and guinea pig anti-ACAT were specific for ACAT in Western blots and did not show labeling in cell extracts from macrophages of ACAT−/− mice (data not shown). Rabbit anti-TGN38 antibodies were generously provided by Dr Keith Stanley (Heart Research Institute, Sydney, Australia) and Dr George Banting (University of Bristol School of Medicine, Bristol, England). Rabbit polyclonal anti-Gos28 antibodies were a gift from Dr Thomas Söllner (Memorial Sloan-Kettering Cancer Center, New York, NY) and were prepared as described.23

Methods

Cells

J774A cells (American Type Culture Collection) were maintained in spinner culture in DMEM (high glucose) containing 10% FBS, 292
with a TRITC-conjugated donkey anti–guinea pig IgG diluted at 1:100 (D) or with an Oregon green–conjugated goat anti-rabbit IgG diluted at 1:200 (A), as described in Methods. Antibodies were detected with Oregon green–conjugated goat anti-rabbit IgG diluted at 1:100. Images were acquired by confocal microscopy as 1-μm-thick optical sections. Panel C shows color overlay of panel A (ACAT, green) and panel B (Cy3-Tf, red). Arrows in panel C point out the paranuclear structure containing ACAT, which does not colocalize with Cy3-Tf. For Golgi colocalization, J774A cells were fixed and incubated with a guinea pig anti-ACAT antibody at 1:100 (D), followed by a rabbit anti-Gos28 diluted at 1:250 (E), as described in Methods. Antibodies were detected for 30 minutes at 37°C. They were fixed with 3% paraformaldehyde in PBS for 10 minutes at room temperature.

**Microscopy and Image Analysis**

Cells were observed by use of laser scanning confocal microscope model LSM 410 (Zeiss) with an argon-krypton laser (488- and 568-nm excitation) and a 100× (numerical aperture 1.4) objective (Figures 1 and 2), by use of laser scanning confocal microscope model LSM 510 (Zeiss) with an Argon laser (488 nm) and a HeNe laser (543 nm) and a 63× (numerical aperture 1.4) objective (Figures 3 and 4), or by use of an epifluorescence microscope (model DMIRB, Leica Inc) with a 63× (numerical aperture 1.32) objective and a cooled CCD camera (Frame Transfer Pentamax with a 512×512 back-thinned EEV chip, model 512EF TB, Princeton Instruments; Figures 5 and 6). Digital images were analyzed by using Metamorph (Universal Imaging), and figures were prepared by using Adobe Photoshop. The intensity value corresponding to the background fluorescence, determined as the average intensity of a blank field, was subtracted from each original image. For double-labeling experiments, the intensity corresponding to the FITC or the Oregon green fluorescence leaking into the TRITC or the Cy3 channels (crossover) was mathematically determined and subtracted from the corresponding TRITC or Cy3 images as described.27 For Figure 2 a 2×2-pixel low-pass filter, resulting in smoothing the image outlines, was applied to each image by using Metamorph.

**Results**

**Characterization of a Guinea Pig Anti-ACAT Antibody**

We previously described the localization of ACAT in mouse peritoneal macrophages by immunofluorescence and confocal microscopy with the use of a rabbit anti-ACAT antibody.15 We showed that in addition to the ER, ACAT was also concentrated in a paranuclear location. Because many of the available organelle-specific antibodies were raised in mice (and would not recognize the murine proteins) or in rabbits, it was difficult to identify the ACAT-containing compartment by immunofluorescence. To facilitate the identification of this compartment, we used in the present study a newly created guinea pig anti-ACAT antibody. In an indirect immunofluorescence study using the J774A mouse macrophage-like cell line, the guinea pig anti-ACAT antibody generated bright labeling (Figure 1B) compared with the dim...
and diffuse fluorescence observed with a nonimmune guinea pig serum (Figure 1A). ACAT was found in the nuclear envelope and in a reticular pattern, consistent with a predominantly ER localization (Figure 1B). The paranuclear compartment previously described for mouse peritoneal macrophages is also seen in J774A cells, but in many cells, it is more prominent than we had previously seen in the primary mouse macrophages (Figure 1B through 1E). In double-labeling studies comparing the distribution of ACAT with protein disulfide isomerase, an ER marker, the nuclear envelope and the reticular pattern were labeled with both antibodies, but the paranuclear spot was not labeled with anti–protein disulfide isomerase (data not shown). To check that the guinea pig anti-ACAT labeled the same compartments as the previously characterized rabbit anti-ACAT, we carried out a double-label immunofluorescence with the 2 anti-ACAT antibodies (Figure 1C through 1E). The rabbit (Figure 1C) and the guinea pig (Figure 1D) antibodies gave the same pattern in J774A cells (Figure 1E), confirming the specificity of the guinea pig antibody and allowing us to use it for the double-labeling experiments required to characterize the paranuclear compartment containing ACAT. It is difficult to quantify the fraction of ACAT in the paranuclear compartment, but it is evident that ACAT is highly enriched in the paranuclear region in many cells. A rough estimate is that >10% of the ACAT may be in this paranuclear region.

Characterization of the Paranuclear Compartment Containing ACAT in Macrophages

We tested whether the paranuclear ACAT compartment was part of the endocytic pathway or of the Golgi complex. In the endocytic pathway, late endosomes and the ERC are at least partly concentrated near the nucleus.19 To examine the possible presence of ACAT in endocytic organelles on the pathway to lysosomes, we incubated the cells with FITC-α2M for 15 minutes (data not shown). Sorting endosomes and the ERC were labeled by incubating cells with Cy3-Tf for 30 minutes (Figure 2B). ACAT was then detected by confocal microscopy after indirect immunofluorescent labeling with use of the rabbit antibody (Figure 2A). We found that organelles containing FITC-α2M do not contain ACAT (data not shown). In particular, the paranuclear structure that contained ACAT did not colocalize with FITC-α2M–labeled endosomes, even though they were sometimes in proximity (data not shown). The ERC, which is a centrally located concentration of transferrin, was often very close to the paranuclear structure containing ACAT, but on close examination, the 2 labels did not align precisely (arrows in Figure 2C). These data indicate that the ACAT-containing compartment is in proximity to the ERC but that the ACAT did not colocalize precisely with any of the endocytic tracers.

The ERC is often close to the Golgi complex.28 To determine the relative localization of ACAT compared with the Golgi complex, we observed cells by confocal micros-
copy after double immunolabeling with the use of guinea pig anti-ACAT antibody (Figure 2D) and a rabbit antibody specific for Gos28 (Figure 2E), a membrane protein present in all Golgi cisternae (cis, medial, and trans Golgi) but not in the TGN.24 We observed that the paranuclear structure containing ACAT was often surrounded by a structure containing Gos28 but did not colocalize with it (Figure 2F), indicating that ACAT did not reside in the Golgi cisternae. In confirmation of this, we found that the overall morphology and intensity of the labeling associated with ACAT was similar in control cells and in cells treated with 5 μg/mL brefeldin A, which causes a redistribution of Golgi membranes29 (not shown).

The cells were then examined for the relative position of ACAT and TGN by confocal microscopy after double immunolabeling with the use of guinea pig anti-ACAT antibody and a rabbit antibody specific for TGN38, an integral membrane protein located in the TGN30 (Figure 3). In a majority of cells, the paranuclear structure containing ACAT was in proximity to part or all of the paranuclear structure concentrated in TGN38. In some cells, concentrations of ACAT could not be resolved from some TGN elements at the resolution of optical microscopy. However, in most cells, a small difference between the distributions of ACAT and the TGN could be seen, especially when 3D reconstructions of confocal images were analyzed. In some cases, the ACAT-rich elements could be seen surrounding elements of the TGN (arrows in Figure 3A through 3C).

To clarify the relationship between ACAT distribution and the TGN, we treated cells with nocodazole. The disruption of microtubules causes a dispersal of the Golgi apparatus and TGN,31 and this would make it easier to see whether a significant amount of ACAT was distributed in the TGN elements. As shown in Figure 4, in J774 cells treated with nocodazole, there was only a small fraction of the TGN-38 spots that also were labeled with ACAT. This indicates that ACAT is close to the TGN but not in it.

**ACAT Distribution in CHO Cells**

To determine if the localization of ACAT near to the TGN and the ERC was specific for macrophages, we localized ACAT in TRVb1/TacTGN38 cells, a CHO-derived cell line that expresses the human transferrin receptor and also expresses a TacTGN38 chimeric protein that has a steady-state distribution mainly in the TGN.26 We found that there was frequently a strongly labeled paranuclear ACAT localization in the TRVb1/TacTGN38 cells. This central localization was typically close to the ERC (not shown) and to the TGN, which was labeled with an anti-Tac antibody that recognized TacTGN38 (Figure 5A through 5C). When the cells were treated with nocodazole, elements of the TGN were dispersed throughout the cell, and there was no significant association of ACAT staining with these dispersed TGN elements (Figure 5D through 5F). These data indicate that ACAT distribution in CHO cells is similar to macrophages, with a significant paranuclear distribution that is close to the TGN but not in it.

Taken together, the data indicate that a significant fraction of ACAT is very close to the TGN and also in proximity to the ERC in macrophages and fibroblasts. However, ACAT does not seem to actually reside in either of these organelles.

**DHE in J774 Cells**

DHE is a fluorescent naturally occurring sterol that is distributed in biological membranes in a manner similar to that of cholesterol.32,33 We have shown previously that in
TRVb-1 cells, DHE labels the ERC and the TGN in addition to the plasma membrane. As shown in Figure 6, DHE is distributed into a paranuclear compartment in J774A cells that very closely overlaps the ERC, which is labeled with transferrin. This region of the cell is also in proximity to the TGN. This shows that the paranuclear concentration of ACAT is in proximity to the cholesterol-rich membranes of the TGN and the ERC.

Discussion

Cholesterol plays an important role in the activation of ACAT, but the mechanisms involved remain unclear. A significant increase in cholesterol esterification activity can be achieved without large increases in the amount of ACAT protein in cells. It has been proposed that cholesterol in membranes can act as an allosteric regulator of ACAT activity. An alternate mechanism for increasing cholesterol esterification would be to increase the rate or efficiency of delivery of cellular cholesterol to ACAT. Evaluation of the importance of such a regulatory mechanism requires a knowledge of the localization of ACAT and the mechanisms for the transport of cholesterol through the cell to ACAT.

Subcellular fractionation studies in liver have shown that most ACAT cofractionates with ER, although some ACAT has been found to copurify with other membranes, such as mitochondria-associated membranes in rat hepatocytes. With the recent availability of antibodies that are specific for ACAT, immunolocalization studies have become possible. Immunofluorescence localization of ACAT in melanoma cells was consistent with an ER localization. In a previous immunofluorescence study in macrophages, we found that ACAT was mostly located in the ER but that there was an enrichment of ACAT in a paranuclear site that was not enriched with other ER markers, such as protein disulfide isomerase. We also found a small fraction of ACAT on the surface of nonadherent macrophages, but surface ACAT was not detected on adherent macrophages. In the present study, we carried out a characterization of the paranuclear ACAT in macrophages by use of immunofluorescence and confocal microscopy.
We observed that ACAT was not enriched in sorting endosomes or late endosomes. This is significant because lipoprotein degradation and cholesterol ester hydrolysis would begin in late endosomes, and close association between ACAT-containing membranes and these organelles would potentially provide a rapid intracellular transport route for lipoprotein-derived cholesterol to ACAT. The lack of such an association is consistent with biochemical studies that have indicated that most lipoprotein-derived cholesterol passes through the plasma membrane before esterification by ACAT.5,11–13

A large fraction of internalized lipids and membrane proteins pass through a subcompartment of the early endosomes, the ERC, which is concentrated around the microtubule-organizing center in many cell types.19 The ERC is a major intracellular site of cholesterol in CHO cells,9 and we have shown in the present study that DHE, a fluorescent cholesterol analogue, is enriched in the ERC in J774 macrophages. Comparing the ACAT distribution with transferrin, we found that the ERC is often close to the paranuclear concentration of ACAT but that the 2 are not coincident.

The Golgi apparatus is also near to the microtubule-organizing center, and the cis Golgi is engaged in extensive bidirectional transport with the ER. It seemed possible that some ACAT might cycle through the cis Golgi. However, there was not a close association between cis and medial Golgi cisternae and ACAT. The lack of effect of brefeldin A on the paranuclear ACAT distribution is consistent with the conclusion that ACAT is not in cis or medial Golgi cisternae. In contrast, there was a very close association in several cells between the paranuclear ACAT concentration and the TGN. Close examination of 3D confocal images indicates that the paranuclear ACAT is in proximity to but does not colocalize with TGN markers. This conclusion is supported by immunofluorescence localization in nocodazole-treated cells, which shows that most TGN38-positive compartments do not contain detectable ACAT. A recent electron microscopic (EM) immunolocalization of ACAT in human macrophages also found that ACAT was not in the Golgi elements.35

All of our results are consistent with the enrichment of ACAT in part of the ER that is in proximity to the TGN and the ERC, although we cannot rule out the possibility that the paranuclear ACAT is in another unidentified compartment. EM immunolocalization of ACAT in human macrophages indicated that most ACAT was in structures that were ER.35 Close association of ER and trans-Golgi elements was suggested from EM studies many years ago.36 More recently, high-resolution 3D tomographic reconstruction of the TGN obtained by multiple tilt angle observations with high-voltage EM have shown that some ER membranes are in very close apposition to parts of the TGN and actually appear as part of the Golgi stacks.37 These EM studies did not determine whether these ER membranes were functionally specialized, but we speculate that such membranes might be enriched in ACAT.

A concentration of ACAT in ER membranes (or in another organelle) near to the ERC and the TGN could play a critical role in delivering cholesterol to ACAT. The ERC and the TGN are major intracellular sites of free cholesterol. The catalytic site of ACAT-1, the form of ACAT in macrophages, likely faces the cytosol,38,39 so its localization near the ERC and TGN would place the active site near to cholesterol-rich organelle membranes. A large fraction of internalized lipids pass through the ERC and are recycled back to the cell surface,40 so ACAT in the paranuclear region could respond rapidly and effectively to changes in cholesterol levels in the plasma membrane. This would be in agreement with biochemical studies showing that vesicular transport is required for the esterification of cholesterol derived from the plasma membrane pool.12,41,42 The TGN also receives vesicular traffic from the recycling compartment26,43 as well as from late endosomes,44–47 and traffic from both of these organelles may be a route for delivery of cholesterol to the TGN.

The presence of an enrichment of ACAT close to 2 major cholesterol-rich organelles that engage in extensive bidirectional traffic with the plasma membrane provides a simple way for the cell to deliver cholesterol to ACAT without requiring any specialized delivery mechanisms. The lipid constituents of the ERC are equilibrated with the plasma membrane by membrane traffic pathways with a half-time of 8 to 30 minutes in various cell types.19 This extensive membrane traffic would easily be sufficient to account for delivery of cholesterol to ACAT.

In addition to the well-characterized division into rough and smooth regions, it is apparent that the ER has other specializations, such as exit sites for protein export and, in some cells, Ca2+ sequestration and release.48,49 Recently, it has been shown that on cholesterol loading, the morphology of ACAT-containing compartments in human macrophages changes so that the structures appear to be small vesicles in single EM thin sections.35 These small vesicle profiles also contain GRP 78, a typical ER marker, indicating that a reorganization of part of the ER has occurred in response to cholesterol loading. The paranuclear concentration of ACAT described in the present study may represent another functional regional specialization of the ER. It will be important to understand how this specialization is established and maintained so that its functional roles can be tested.

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