Hydrolysis of Lipid Droplets in Acid Cholesteryl-Esterase-Deficient Fibroblasts

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ABSTRACT. The mechanisms of hydrolysis and accumulation of cholesteryl oleate-lipid droplets prepared in vitro were studied in acid cholesteryl-esterase-deficient fibroblasts (GM00863, GM03111).

Acid cholesteryl esterase activity was reduced in both GM00863 and GM03111 (8.9% and 17.4% of the normal level, respectively), while neutral cholesteryl esterase activity was highly stimulated in GM03111. The hydrolysis of [14C]-cholesteryl oleate-lipid droplets in GM00863 was almost as efficient as in normal cells, while that in GM03111 was highly stimulated. When viewed by polarized microscopy the lipid droplets which had accumulated in the mutant cells showed anisotropic liquid crystalline structures. As in normal cells, some of these lipid droplets were observed by transmission electron microscopy as membrane-free lipid inclusion bodies in the cytoplasm. These results suggest that lipid droplets internalized into phagolysosomes of these mutant cells transferred to the cytoplasm, and were hydrolyzed there probably by neutral cholesteryl esterase.

A striking feature of atherosclerosis is the marked accumulation of lipids, especially cholesteryl esters, in the arterial wall (16, 20). These lipids can be observed by polarized microscopy as intense anisotropic droplets (8, 12). Ultrastructural observations have shown that the lipids accumulate in cells in two distinct states (6, 9, 23, 24), either as lipid droplets surrounded by a limiting membrane in the phagolysosomes or as droplets without a limiting membrane in the cytoplasm.

Cholesteryl ester-lipid droplets are originally derived from lipoproteins in the blood stream (2, 3). Arterial cells take up lipoproteins by endocytosis, hydrolyze the cholesteryl esters with lysosomal acid cholesteryl esterase, and re-esterify the free cholesterol with microsomal acyl-CoA: cholesterol acyltransferase (ACAT) (4, 5, 10). The cholesteryl esters accumulate in the cytoplasm as lipid inclusion bodies. In atherosclerosis, the lipid droplets, derived from ruptured foam cells, are deposited in the extracellular connective tissue space (1), and recaptured by either macrophages or modified smooth muscle cells (14, 28, 29). In previous studies, we have found that, in peritoneal macrophages, cholesteryl oleate-lipid droplets prepared in vitro are transferred directly from phagolysosomes to the cytoplasm either with or without partial hydrolysis in lysosomes (18), not via the re-esterification in microsomes. We designated this second pathway the 'vesicular pathway'.

In this study, we used human fibroblasts deficient in acid cholesteryl esterase to examine how these cells take up the lipid droplets prepared in vitro and how they metabolize them. We discuss the possibility that the lipid droplets are metabolized via the 'vesicular pathway' in these mutant cells.

MATERIALS AND METHODS

Materials. Two cell strains of cholesteryl-esterase-deficient human fibroblasts (GM00863 and GM03111) and normal human fibroblasts (GM00011) were purchased from the Human Genetic Mutant Cell Repository (National Institute of General Medical Science, NJ), and maintained in Dulbecco's Modification of Eagle's Medium (D'MEM, Flow Labs., McLean, VA) containing 10% fetal calf serum (Flow Labs., McLean, VA). [14C]cholesteryl oleate was obtained from New England Nuclear (Boston, MA).

Assay of cholesteryl esterase activity. The fibroblasts were collected with a rubber policeman and washed 3 times with cold phosphate-buffered saline (PBS). The cells were homogenized with a Teflon-glass Potter Elvehjem homogenizer, or sonicated using a Branson Sonifier with a microprobe and then centrifuged (2,500 x g, 10 min). The resulting supernatant was used for the enzyme assay.

Cholesteryl esterase activity was measured with 4-methylumbelliferol (MU) oleate (Koch-Light Lab., Colnbrook, UK) as
the substrate in 0.1 M acetate buffer (pH 3–5.5), 0.1 M sodium phosphate buffer (pH 6–7), or 0.1 M Tris-HCl buffer (pH 7.5–9) (15).

Preparation of cholesteryl oleate lipid droplets. [14C]cholesteryl oleate-lipid droplets were prepared as described previously (26). Briefly, cholesteryl oleate (20 μmole) and 20 μl of [14C]cholesteryl oleate (2.2 GBq/μmole, 3.7 MBq/ml) were sonicated in distilled water for 4 min at 80°C and suspended in 0.004% bovine serum albumin fr. V (Sigma, St. Louis, MO).

Uptake and hydrolysis of [14C]cholesteryl oleate-lipid droplets by fibroblasts. Fibroblasts were cultured in a 60 mm² plastic culture dish (Corning, New York, NY), and incubated with [14C]cholesteryl oleate-lipid droplets (1 μmole/dish, 2.5 × 10⁸ dpm/dish) in D'MEM containing 1% fetal calf serum using the method involving inverted petri dishes, as previously described (7). After removal of the medium, the cells were washed 4 times with PBS and sonicated using a Branson Sonifier. The lipid fraction was extracted with chloroform-methanol (2:1, v/v), and free and esterified cholesterol were separated on silica gel thin layer chromatogram sheets (Eastman-Kodak, Rochester NY) (petroleum ether : ether, 80:20). The radioactivity in each fraction was measured with a liquid scintillation counter (Aloka 300, Tokyo, Japan). To measure the efflux of [14C]cholesterol into the medium, fibroblasts were precultured with [14C]cholesteryl oleate-lipid droplets for 18 h at 37°C, and after washing 5 times with PBS, the cells were incubated in D'MEM containing 10% fetal calf serum without lipid droplets. The radioactivity in both the medium and the cells was measured. Protein content was determined using BCA protein assay reagent (Pierce Chemical Co., Rockford, IL).

Electron microscopy. Monolayers of fibroblasts were fixed with Karnovsky's fixative (17) and then postfixed with 1% OsO₄ in 0.1 M sodium phosphate buffer (pH 7.5). The fixed cells were stained with 1% uranyl acetate en bloc, followed by conventional techniques for transmission electron microscopy.

RESULTS

Acid cholesteryl-esterase-deficiency in mutant fibroblasts. Cholesteryl esterase activity was measured at pH 3–9 with 4MU-oleate as the substrate. The acid cholesteryl esterase activity measured at pH 4.5 in mutant fibroblast GM00863 and GM03111 was 8.9% and 17.4% of the normal level, respectively (Fig. 1). In GM00863 cells, the activity of neutral cholesteryl esterase at pH 7.5 was the same as in normal cells, whilst in the other mutant, GM03111, the activity of neutral cholesteryl esterase was 9.4 times higher than in normal fibroblasts.

Uptake and hydrolysis of [14C]cholesteryl oleate-lipid droplets. When mutant cells were incubated with cholesteryl oleate-lipid droplets (1 μmole/60 mm² dish) for 24 h, an accumulation of lipid droplets with anisotropic crystalline structure was observed (data not shown). The rate of internalization of [14C]cholesteryl oleate-lipid droplets into the mutant cells was almost the same as in normal cells (Table I). Under these conditions, the hydrolysis of [14C]cholesteryl oleate-lipid droplets was investigated (Fig. 2). In the GM00863 cell line the lipid droplets were hydrolyzed as efficiently as in normal cells. Furthermore, GM03111, which showed higher neutral esterase activity, hydrolyzed the lipid droplets more efficiently than did normal cells.

During the course of this incubation, the release of free cholesterol into the medium seems to be minimal, as evidenced by the pulse-chase experiment. Namely, the efflux of [14C]-labelled materials into the medium was measured after the cells had been preincubated with

<table>
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<tr>
<th>Table I. UPTAKE OF [14C]CHOLESTERYL OLEATE-LIPID DROPLETS BY FIBROBLASTS.</th>
<th>(nmole/mg cell protein)</th>
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<tbody>
<tr>
<td></td>
<td>12 h</td>
</tr>
<tr>
<td>GM00011</td>
<td>27.7±9.2</td>
</tr>
<tr>
<td>GM00863</td>
<td>25.8±0.1</td>
</tr>
<tr>
<td>GM03111</td>
<td>30.6±2.8</td>
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Fibroblasts were incubated with [14C]cholesteryl oleate lipid droplets (1 μmole/dish, 2.5 × 10⁸ dpm/dish) for 12 h and 24 h. The cells were homogenized and the radioactivity in the cells was measured by liquid scintillation counting. The data are mean±SD (n=3).
Fig. 2. Hydrolysis of [14C]cholesterol oleate-lipid droplets in fibroblasts.
Fibroblasts were incubated with [14C]cholesterol oleate lipid droplets (1 µmole/dish, 2.5 x 10^5 dpm/dish) for 12, 24, 38, and 46 hours. After the medium was removed, the cells were homogenized and the amount of [14C]-labelled free and esterified cholesterol was measured as described in Materials and Methods. The amount of free cholesterol is indicated as a percentage of the total cholesterol in the cells. Cells: Normal fibroblast cells (GM00011) (○), mutant fibroblast cells, (GM00863) (●), (GM03111) (■).

Fig. 3. Efflux of [14C]-free and esterified cholesterol into the medium.
Fibroblast cells were precultured with [14C]cholesterol oleate lipid droplets (1 µmole/dish, 2.5 x 10^5 dpm/dish) in D'MEM containing 1% fetal calf serum for 18 h at 37°C, and after washing, the cells were incubated for 6 h and 24 h in the absence of lipid droplets. The amount of [14C]-cholesterol and esterified cholesterol in the cells (solid lines) and medium (dotted lines) was measured by liquid scintillation counting. Cells: Normal fibroblast cells (GM00011) (○), (GM00863) (●), (GM03111) (■). Figure 4B inset shows a high-magnification view of Lp2, which is not surrounded by a limiting membrane. These results suggest that the internalized lipid droplets escape from phagolysosomes and accumulate in the cytoplasm as non-membranous lipid inclusion bodies even in the acid cholesteryl-esterase-deficient fibroblasts.

DISCUSSION
During the development of atherosclerosis, arterial wall cells accumulate cholesteryl ester-rich anisotropic lipid droplets in the cytoplasm. It has been demonstrated that the lipoprotein-derived cholesteryl esters are internalized via receptor-mediated endocytosis, hydrolyzed by lysosomal acid cholesteryl esterase, and the free cholesterol is re-esterified by microsomal ACAT (4, 5, 10). Previously, we have proposed that lipid droplets accumulated in the cytoplasm via a ‘vesicular pathway’, during which lipid droplets are transferred from phagolysosomes directly to the cytoplasm (7, 18, 22). In this proposed pathway, anisotropic lipid droplets first internalized in endosomes or phagolysosomes escaped from these compartments without under going hydrolysis. In atherosclerotic lesions in vivo, anisotropic lipid droplets have also been observed in the connective tissue space (1), and these exogenous lipid droplets are taken
Fig. 4. Electron micrographs of lipid droplet-laden fibroblasts. Normal and mutant fibroblast cells were incubated with lipid droplets (1 μmole/dish). A; normal fibroblast cells incubated with lipid droplets for 6 h (×29,000). (Inset; high magnification view, × 47,000) B; acid cholesteryl-esterase-deficient fibroblasts (GM03111) incubated with lipid droplets for 18 h (×14,000). (Inset; high-magnification view, × 37,600). Lp1; lipid droplets surrounded by a limiting membrane. Lp2; lipid droplet without a limiting membrane. Ly; lysosomal membrane surrounding lipid droplets.

We have also shown that, even in these mutant cells, internalized lipid droplets accumulate in the cytoplasm as membrane-free lipid inclusion bodies. One possible explanation for how these inclusion bodies accumulate in the cytoplasm is that the lipid droplets are transferred from phagolysosomes to the cytoplasm via a 'vesicular pathway'. Namely, the exogenous lipid droplets were endocytosed by fibroblasts, incorporated in the endosome/lysosome compartment, and then transferred to the cytoplasm without being hydrolyzed. An alternative explanation is that the exogenous lipid droplets are internalized directly into the cytoplasm through the plasma membrane. In our previous studies, we found that most cells.
lipid droplets in the macrophages were surrounded by a limiting membrane after 24 h incubation, and that the limiting membrane disappeared with further incubation (18). It is shown in this study that fibroblasts contain lipid droplets surrounded by a limiting membrane at an early time point (Fig. 4, Lp1). The results suggest that lipid droplets internalized into the fibroblasts by endocytosis are first localized in the endosomal or lysosomal compartments, and then transferred to the cytoplasm. It is possible that some of the lipid droplets without a limiting membrane (Lp2) are re-esterified cholesterol derived from the microsomal pathway. However, since the mutant cells had very low cholesteryl esterase activities (as shown in Fig. 1), it is less likely that the cholesteryl oleate hydrolyzed in lysosomes was re-esterified in the microsomes. But the pathway, during which the lipid droplets transferred to the cytoplasm were hydrolyzed by cytoplasmic neutral esterase, and then re-esterified by microsomal ACAT, might be involved in the accumulation of Lp2 in the cytoplasm.

Hydrolysis of cholesteryl oleate in LDL was reported to be suppressed in these mutant fibroblast cells (19, 25). We also investigated the hydrolysis of [14C]-cholesteryl oleate labelled LDL in the mutant cells and found that, in GM03111, which hydrolyzed lipid droplets much more efficiently than the second mutant strain, GM00863, or normal fibroblasts, the hydrolysis of LDL was not stimulated, but stayed at the same level as in GM00863 (data not shown). The discrepancy between the hydrolysis of cholesteryl esters in lipid droplets and in LDL can be explained by the size and the hydrophobicity of the particles, or the association of cholesteryl esters with proteins. The other possible explanation is that the phagocytotic pathway, by which lipid droplets are internalized into the cells, may be involved in the hydrolysis of the lipid droplets, not in the lysosomes, but in the cytoplasm, since LDL enter cells via the receptor-mediated pathway.

The mechanism by which the lipid droplets transfer from the endosome/lysosome compartment to the cytoplasm without hydrolysis is still unknown. One possible explanation, by analogy to the accumulation of membrane-free vitamin A-rich lipid droplets in the cytoplasm (27), is that the limiting membrane may be disrupted by some mechanism or another. Alternatively, the hydrophobic lipid inclusion bodies may fuse with the endosomal/lysosomal membrane and be transferred to the cytoplasm.

In this study, we suggest that lipid droplets were transferred from phagolysosomes to the cytoplasm via a 'vesicular pathway', and hydrolyzed by cytoplasmic neutral cholesteryl esterase. This pathway may be important in the accumulation of cholesteryl ester in foam cells in atherosclerotic lesions.

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REFERENCES


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