Sequestration of Acetylated LDL and Cholesterol Crystals by Human Monocyte-Derived Macrophages

Howard S. Kruth, Sonia I. Skarlatos, Kathleen Lilly, Janet Chang, and Ina Ifrim

Section of Experimental Atherosclerosis, National Heart, Lung, and Blood Institute, National Institutes of Health, Bethesda, Maryland 20892

Abstract. Monocyte-derived macrophages accumulate and process cholesterol in atherosclerotic lesions. Because of the importance of this process, we examined the interaction of cholesterol crystals and acetylated low density lipoprotein (AcLDL) with human monocyte-macrophages in a combined chemical and morphological study. These two forms of cholesterol induced extensive compartmentalization of the macrophage cytoplasm. Unexpectedly, the compartments maintained a physical connection to the extracellular space as demonstrated with ruthenium red staining. The compartments formed through invagination of the top surface of the macrophage plasma membrane. Some cholesterol crystals and AcLDL were sequestered within these surface-connected compartments for up to five days in the case of the crystals and for one day in the case of AcLDL.

Pulse-chase studies of fractionated macrophages indicated that \(^{3}H\) cholesterol redistributed from the surface-connected compartments into lysosomes (where the cholesterol remained unesterified) and into lipid droplets (where the cholesterol was stored as cholesteryl ester). Intracellular uptake and esterification of cholesterol was blocked by cytochalasin D. However, once cholesterol was sequestered in the surface-connected compartments, subsequent esterification of the cholesterol could not be inhibited by cytochalasin D. Apolipoprotein E was localized within the surface-connected compartments by immunogold labeling suggesting a possible function for this protein in the processing of lipid taken up through the sequestration pathway.

Removal of microcrystalline cholesterol from the medium resulted in release of most of the accumulated cholesterol microcrystals from the macrophages, as well as disappearance of the surface-connected compartments. Thus, sequestration is a novel endocytic mechanism in which endocytic compartments remain connected to the extracellular space. This differs from phagocytosis where endocytic vacuoles rapidly pinch off from the plasma membrane. Sequestration provides a means for macrophages to remove substances from the extracellular space and later release them.

Materials and Methods

Isolation of Human Monocytes

We prepared human monocytes from acid citrate/dextrose-treated plasma (2.5 g of dextrose, 2.2 g of sodium citrate, and 0.73 g of citric acid per 100 ml of plasma) that was enriched with mononuclear cells by the process of mononuclear pheresis (model CS-3000, Fenwall Inc., Ashland, MA). Mononuclear cells were further purified by the method of Boyum (1976). 4-ml aliquots of the mononuclear cell-enriched plasma were diluted with 12 ml...
of Ca²⁺ and Mg²⁺-free Dulbecco's phosphate-buffered saline (DPBS) (310-4190 AJ; GIBCO BRL, Gaithersburg, MD) in 50-mL conical polypropylene tubes (Falcon Plastics, Franklin Lakes, NJ). 12 ml of Ficoll-hypaque (36427; Organon Teknika Corporation, Rockville, MA) was then pipetted under the diluted mononuclear cell-enriched plasma. The layered preparation was centrifuged for 7 min at 1,900 g and 22°C. The upper plasma layer was removed and the band of mononuclear cells was harvested.

The mononuclear cells were diluted with an equal volume of Ca²⁺ and Mg²⁺-free DPBS, mixed by inversion, and centrifuged for 10 min at 300 g and 22°C. The pellet was resuspended in 40 ml of Ca²⁺- and Mg²⁺-free DPBS and centrifuged for 7 min at 300 g and 22°C. The pellet was resuspended in 5 ml of RPMI 1640 (12-602-54; Flow Laboratories Inc., McLean, VA) containing 10% pooled human AB. heat-inactivated serum (34004-1; Pel-Freez, Rogers, AZ). Glutamine was added to all RPMI medium at a final concentration of 0.33%.

The purified mononuclear cells were counted with a Coulter counter (model ZM, Coulter Corp., Hialeah, FL). The cell suspension was diluted to 5 × 10⁶ mononuclear cells/mL in RPMI 1640 containing 10% human serum. The distribution of monocytes, lymphocytes, and platelets was determined from the Coulter Counter size analysis of cells. Typically 30% of the mononuclear cells were monocytes and 70% were lymphocytes. The cell suspensions also contained about four platelets per mononuclear cell.

Culture of Human Monocyte-derived Macrophages

10 × 10⁶ mononuclear cells were seeded into individual 35-mm diameter wells of six-well cluster dishes (606C, Plastek-C; Tekmat, Ashland, MA) and cultured as described previously (Skarlatos et al., 1992). For electron microscopy experiments, 5 × 10⁶ mononuclear cells were seeded into each well of a four-well plastic (Permanox) chamber-slide (177437, Lab-Tek; Nunc, Naperville, IL). All rinsing and incubation solutions were 1 ml for wells of cluster dishes and 2 ml for wells of six-well cluster dishes.

Macrophage cultures were then fed RPMI 1640 containing 10% pooled hu-

man AB, heat-inactivated serum (3 ml for wells of cluster dishes and 1 ml for wells of six-well cluster dishes). Monocyte-macrophage cultures were then fed RPMI 1640 containing 10% pooled human AB, heat-inactivated serum (3 ml for wells of cluster dishes and 1 ml for wells of six-well cluster dishes).

After seeding, mononuclear cells were incubated for 1 h at 37°C during which time the monocytes attached to the well bottom. Then, unattached cells were removed by rinsing each well twice with RPMI 1640. Monocyte-macrophage cultures were then fed RPMI 1640 containing 10% pooled human AB, heat-inactivated serum (3 ml for wells of cluster dishes and 1 ml for wells of six-well cluster dishes) and incubated for 1 wk. The medium was replaced with the same volumes of fresh medium at this time and after the second week in culture. All cell cultures and incubations were carried out in a humidified 37°C incubator maintained with a 5% CO₂/95% air atmosphere.

Incubation of Macrophages with AcLDL and Microcrystalline Cholesterol

On the day experiments were begun, cell cultures were rinsed twice with RPMI medium. Then, macrophages were incubated for designated times in medium without additions or with the indicated concentrations of microcrystalline cholesterol or AcLDL (Skarlatos et al., 1993a). The concentrations of AcLDL are expressed as µg of AcLDL protein per ml of medium. The microcrystalline dispersion of cholesterol (32 mM) was prepared by dissolving cholesterol (>99% pure, C3045; Sigma Chem. Co., St. Louis, MO) in ethanol at 37°C. The microcrystalline dispersion of cholesterol was added to serum-free RPMI 1640 medium (37°C) to produce a final cholesterol concentration of 50 to 400 µg/ml as indicated. RPMI 1640 medium was vortexed during the addition of the cholesterol solution. AcLDL was prepared as described by Basu et al. (1981), and was examined by negative staining as previously described (Kruth et al., 1994). All medium contained 1% (vol/vol) penicillin-streptomycin (600-5140 AG, 10,000 U/ml penicillin and 10,000 µg/ml streptomycin) and 2% glutaraldehyde in 0.1 M Na-cacodylate buffer (CB) (pH 7.4).

After fixation of lipids and removal from thin layer plates, 'H-unesterified cholesterol and 'H-esterified cholesterol samples were quantitated with a scintillation counter. The lysosomal marker enzyme, β-hexosaminidase, was assayed fluorometrically using 4-methylumbelliferyl-2-acetamido-2-deoxy-β-D-glucopyr

anose (M2133; Sigma) as substrate as described by Stirling (1984). The recovery during Percoll density gradient fractionation for all analytes was >85%.

Determination of Lipid Content of Macrophages

Following incubations, monocyte-derived macrophage cultures were rinsed three times with DPBS containing 0.35% bovine serum albumin, and then three times with DPBS without BSA. Finally, the cells were scraped into 2 ml double distilled water and kept frozen at -70°C until chemical analyses were performed.

For chemical analyses of macrophages, cell samples were thawed and sonicated on ice for 30 s at a power setting of 25 watts (sonifier no. 20; Branson Ultrasonics Corp., Danbury, CT). 100 µl of each cell sample was analyzed for protein content by the method of Lowry et al. (1951). Bovine serum albumin was used as a standard. 1 ml of each cell sample was extracted with chloroform/methanol (2:1, vol/vol) according to Folch et al. (1957) for later determination of lipids. Unesterified and esterified cholest

erol were determined enzymatically with the fluorometric method of Gam

ble et al. (1978). Total phospholipid phosphorus was determined by the method of Bartlett (1959). All samples were analyzed in duplicate.

Percoll Density Gradient Fractionation of Macrophages

Subcellular fractionation of total cell homogenates was carried out essen
tially as described previously (Morand and Kent, 1986). Cultures were rinsed three times with DPBS containing 0.35% bovine serum albumin, and then three times with DPBS without BSA. Following incubations, monocyte-derived macrophage cultures were rinsed three times with DPBS (310-4040 AJ; GIBCO BRL) containing 0.35% BSA and then three times with DPBS without BSA. Finally, the cells were scraped into 2 ml double distilled water and kept frozen at -70°C until chemical analyses were performed.

For chemical analyses of macrophages, cell samples were thawed and sonicated on ice for 30 s at a power setting of 25 watts (sonifier no. 20; Branson Ultrasonics Corp., Danbury, CT). 100 µl of each cell sample was analyzed for protein content by the method of Lowry et al. (1951). Bovine serum albumin was used as a standard. 1 ml of each cell sample was extracted with chloroform/methanol (2:1, vol/vol) according to Folch et al. (1957) for later determination of lipids. Unesterified and esterified cholest

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Electron Microscopy of Macrophages

Processing of Macrophages for Routine Electron Microscopy

Macrophage cultures were rinsed three times with DPBS containing 0.35% bovine serum albumin and three times with DPBS. All solutions used for electron microscopy were 2 ml per well. Fixation of monolayers was carried out with 2% glutaraldehyde in 0.1 M Na-cacodylate buffer (CB) (pH 7.4) containing 2 mM CaCl₂ and 0.1 M sucrose for 1 h at 4°C. Monolayers were rinsed three times with 0.05 M CB containing 2 mM CaCl₂ and 0.2 M sucrose and post-fixed with 2% osmium tetroxide for 2 h at 4°C. Monolayers were then rinsed with three 5-min changes of 0.1 M CB at 4°C.

Dehydration of monolayers was carried out at room temperature in 5-min

rises of ascending ethanol concentrations (three changes of 70%, one change of 95%, and three changes of 100%). At this point the upper cham

ber was removed from the slide culture so all four wells of the slide could be processed simultaneously. The slide culture was then process

ed in capped 50-ml polypropylene tubes containing the embedding medium. Cul

tures were infiltrated overnight with EMBid 812 embedding mixture (14120, Electron Microscopy Sciences, Ft. Washington, PA) added 1:1 (vol/vol)
with 100% ethanol. Next, cultures were infiltrated with two changes of EMbed 812 embedding mixture (2 h each change). Slide-cultures were then placed monolayer face down in an embedding mold (70170, Electron Microscopy Sciences) containing fresh EMbed 812 embedding mixture and polymerized 48 h.

After polymerization, embedded monolayers were removed from plastic slides by peeling the polymerized EMbed 812 monolayer away from the slide. Regions of cultures to be sectioned were cut out and reembedded in flat embedding molds (105; Ted Pella Inc., Redding, CA) to produce either en face (i.e., parallel to the culture surface) or cross sections (i.e., perpendicular to the culture surface) as indicated. When counterstaining was carried out this was done with 2% uranyl acetate (2 min) and 0.4% lead citrate (7 min). Sections were examined at 60 kV in a Philips 400 microscope.

**Photoconversion of Dil-labeled AcLDL.** Cultures were rinsed six times as described for routine electron microscopy and then fixed in DPBS containing 0.2% glutaraldehyde plus 2.5% paraformaldehyde for 1 h at room temperature. After fixation cultures were rinsed with three five-min DPBS rinses. Next, the fluorescent Dil-AcLDL was localized by a photoconversion reaction as described by Tabas et al. (1990). In this procedure, diaminobenzidine is reduced to an insoluble electron dense product during photolilumination of a fluorescent probe (Maranto, 1982; Sandell and Masmial, 1988). Fresh photoconversion solution was prepared by dissolving 1.5 mg/ml of diaminobenzidine (Sigma) into 0.1 M Tris–HCl (pH 7.5). Photoconversion solution was added to cultures that were then exposed to ultraviolet light. A Leitz Orthoplan microscope equipped with a 10× brightfield objective and 100 W mercury-arc lamp was used to focus the ultraviolet light onto the monolayers using epi-illumination. The ultraviolet light was filtered through a UGI filter supplied with the microscope. After 10-min exposure, photoconversion solution was replaced with fresh photoconversion solution and monolayers were exposed to ultraviolet light another 10 min. A brown spot appeared in the illuminated area.

Following photoconversion, cultures were rinsed three times with 0.1 M Tris–HCl buffer and three times with DPBS. Cultures remained in DPBS overnight at 4°C and then were processed for electron microscopy. Cultures were fixed with 2% glutaraldehyde in DPBS for 1 h at room temperature, rinsed twice with DPBS, and post-fixed with 2% osmium tetroxide for 2 h at room temperature. After three 5-min rinses with dH2O, cultures were dehydrated in acetone (three changes of 70%, one change of 95% and three changes of 100%). For the photoconversion procedure, the upper chamber of the culture dishes was removed from the slide-culture before dehydration because it was not resistant to acetone. The slide-cultures were infiltrated overnight in a 1:1 (vol/vol) mixture of EMbed 812 and 100% acetone and then processed as described for routine electron microscopy.

**Determination of Extracellular Space with Ruthenium Red.** Macrophages were rinsed six times as described for routine electron microscopy except as noted otherwise. Cultures were fixed in 2% glutaraldehyde in 0.1 M CB for 30 min and then placed in the same fixative containing 0.15% ruthenium red (2 rain; Sigma) (Luh, 1978). For an additional 30 min, two fixation steps were carried out both at room temperature and at 4°C to compare results. All subsequent steps were carried out at room temperature. Cultures were rinsed three times with 0.1 M CB, and post-fixed for 3 h in 2% osmium tetroxide in 0.1 M CB containing 0.15% ruthenium red. Then, cultures were rinsed three times in 0.1 M CB before they were dehydrated and embedded as described for routine electron microscopy.

**Immunogold Labeling of Cathespin D and Apolipoprotein E.** Following incubations, monolayers were rinsed six times as described for routine electron microscopy, and then two times with pH 7.4 wash buffer (0.1 M sodium phosphate containing 0.05 mM CaCl2 and 4% sucrose). The immunogold labeling method used was adapted from the procedure described by Berryman and Bodewald (1990). All processing steps were carried out at 4°C. Following incubations, monolayers were fixed for 1 h with 4% paraformaldehyde, 0.5% glutaraldehyde, 0.2% picric acid, 4% sucrose, and 0.05 mM CaCl2 in 0.1 M sodium phosphate buffer (pH 7.4).Fixative was removed from monolayers with four 5-min rinses of wash buffer. Cells were then quenched for 1 h with wash buffer containing 50 mM NaN3. After four 15-min rinses in 0.1 M maleate buffer with 4% sucrose (pH 6.5), macrophages were stained for 30 rain with 2% uranyl acetate in the maleate-sucrose buffer (pH 6.0). Then, macrophages from three wells were scraped from the culture surface and collected into a 15 ml polycarbonate tube. Cells were pelleted (500 g for 5 min) and resuspended successively in 5 ml of 50, 70, and 82% ethanol solutions. Cells were centrifuged and resuspended a second time in 1 ml of 82% ethanol and transferred to 1.5-ml microcentrifuge tubes. After macrophages were pelleted in these tubes for 4 min at 500 g, they were resuspended in 1 ml of 2:1 LR White resin (17411; Polysciences Inc., Warrington, PA) and 82% ethanol mixture. Cells were kept in this mixture for 30 min before they were pelleted for 8 min at 500 g and resuspended in 1 ml of 100% LR white resin where they remained at least overnight. Then, infiltration of resin was completed with five 60-min changes with 1 ml of fresh LR white resin by pelleting and resuspending the cells as described above. Lastly, the cell pellet (resuspended in a drop of LR white resin) was transferred to a gelatin capsule (size 00). The capsules were filled to the top with fresh LR white resin and capped before polymerization of the resin for 24 h at 57°C.

Gold sections were prepared and mounted on 400-mesh nickel grids. Immunogold labeling was carried out at room temperature except when noted otherwise. First, sections were incubated for 10 min with TBS (50 mM Tris, 150 mM sodium chloride, pH 7.4) containing 1% BSA (AR101, crystalline grade; Chemicon Intl. Inc., Temecula, CA). Sections were then incubated overnight at 4°C with a mouse monoclonal anti-apoipoprotein E antibody purified from ascites fluid by protein A affinity chromatography (clone 21-D3; Biogenesis, Sandown, NH), or a mouse monoclonal anti-cathepsin D antibody in hybridoma supernatant (clone C5; Biogenesis). The anti-apoipoprotein E antibody was used at a concentration of 10 µg/ml and the anti-cathepsin D antibody was used at a dilution of 1:10. Sections were incubated with the same concentration of the purified mouse myeloma protein MOPC 21 (50327, Cappel Labs., Cochranville, PA) as a control for apo- lipoprotein E immunostaining. Sections were incubated with the same dilution of a hybridoma supernatant with specificity towards Aspergillus major glucose oxidase (clone DAK-G09; Dako Corp., Carpenteria, CA) as a control for cathepsin D immunostaining. In addition, other control sections were incubated with 10 µg/ml purified mouse IgG as all monoclonal antibodies were IgG. Lastly it was determined whether or not labeling by the anti-apoipoprotein E antibody could be blocked by preincubation of the antibody with purified apolipoprotein E (178466, Calbiochem-Novabiochem, La Jolla, CA). For this control, 0.05 µg of apolipoprotein E was incubated at room temperature for 30 min with 0.36 µg of diluted anti-apoipoprotein E antibody.

After overnight incubation with specific and control primary antibodies, five 5-min rinses with TBS were used to remove unbound primary antibody. This was followed by a 10-min incubation in TBS containing 1% BSA, and incubation for 2 h with gold-labeled (10 nm) goat anti-mouse IgG + IgM (15736-1; Ted Pella Inc.) diluted 1:10 (vol/vol) in TBS with 0.1% BSA. Five 2-min rinses with TBS and five 2-min rinses with dH2O were used to remove the excess gold-labeled secondary antibody. Then, sections were fixed for 20 min with filtered (0.22 µm pore size) 2% osmium tetroxide, rinsed with dH2O (three 1-min rinses), and counterstained with Venable's (1965) lead citrate for 5 min.

## Results

**Accumulation of Cholesterol by Macrophages Incubated with Microcrystalline Cholesterol or AcLDL.**

Most cholesterol accumulation occurred during the first day of incubation of macrophages with microcrystalline cholesterol. However, the esterified cholesterol content of macrophages progressively increased during incubation with the microcrystalline cholesterol. Most of the accumulation of cholesterol in macrophages induced by AcLDL occurred during the first two days of incubation. In contrast to the predominant accumulation of unesterified cholesterol in macrophages incubated with high levels of microcrystalline cholesterol, most of the cholesterol accumulated in macrophages incubated with AcLDL was esterified. Whereas saturation of cholesterol accumulation occurred when macrophages were incubated with 25 µg/ml of AcLDL, saturation of cholesterol accumulation was not achieved when macrophages were incubated with up to 400 µg/ml of microcrystalline cholesterol. Incubation of macrophages with AcLDL or microcrystalline cholesterol for up to four days did not increase or change the phospholipid content of these macrophages compared to macrophages that were not incubated with cholesterol.
Microcrystalline Cholesterol and AcLDL Induced and Accumulated in Surface-connected Compartments

During accumulation of lipid, both microcrystalline cholesterol and AcLDL induced formation of cytoplasmic compartments that could occupy as much as half the cytoplasmic space of the macrophage. These compartments appeared empty in thin sections of macrophages prepared by routine electron microscopy. Ruthenium red staining of fixed macrophages showed that microcrystalline cholesterol and AcLDL had entered these compartments and that the compartments were in continuity with the extracellular space (Fig. 1). Ruthenium red staining was obtained for macrophages that had been fixed at 4°C or at room temperature before exposure to the ruthenium red.

Incubation of macrophages with 50 µg/ml of microcrystalline cholesterol resulted in slight to moderate compartmentalization of the perinuclear region. Incubation of macrophages with a four- to eightfold higher concentration of microcrystalline cholesterol (200 or 400 µg/ml) resulted in much more extensive compartmentalization (Fig. 1b) and more cholesterol accumulation. Thus, the degree of compartmentalization reflected the degree of cholesterol accumulation. Compartmentalization was observed after only three hours of incubation of macrophages with microcrystalline cholesterol or AcLDL. However, compartments remained up to four days during incubation of macrophages

Figure 1. Delineation of extracellular spaces of monocyte-macrophages with ruthenium red. Monocyte-macrophage cultures were incubated in RPMI 1640 medium without any addition for 4 d (a), with 400 µg/ml microcrystalline cholesterol for 4 d (b), or with 25 µg/ml AcLDL for 1 d (c). Next, cultures were fixed in glutaraldehyde and then exposed to ruthenium red during additional fixation in fresh glutaraldehyde and osmium tetroxide. Monolayers were embedded in situ and sectioned parallel to the culture surface. Note the presence of ruthenium red staining within cytoplasmic compartments induced by incubating monocyte-macrophages with the microcrystalline cholesterol or AcLDL. The plasma membrane of control macrophages showed intense ruthenium red staining, whereas the plasma membrane of macrophages incubated with microcrystalline cholesterol and AcLDL showed reduced ruthenium red staining. Sections were not counterstained. Bars, 1 µm.
with microcrystalline cholesterol, but were not observed past one day for macrophages that were incubated with AcLDL. This was probably due to the lower amount of cholesterol that could be delivered to macrophages with AcLDL compared to the amount of cholesterol delivered to macrophages with microcrystalline cholesterol.

No ruthenium red-stained compartments were present in control macrophages that had been incubated in medium without microcrystalline cholesterol or AcLDL (Fig. 1a). Ruthenium red did stain the external plasma membranes of control macrophages, as well as macrophages incubated with microcrystalline cholesterol and AcLDL. However, the in-

Figure 2. Demonstration of the openings of surface-connected compartments in monocyte-macrophages. Monocyte-macrophage cultures were incubated with 400 μg/ml microcrystalline cholesterol (a and c–g), or 25 μg/ml AcLDL (b) for 1 d. Then, cultures were fixed in glutaraldehyde and exposed to ruthenium red. The monolayers were embedded in situ and sectioned perpendicular to the culture surface. Arrows in a and b indicate compartments (that contained microcrystalline cholesterol or AcLDL) opening at the top surface of the macrophage. Inset in a shows the same opening (at a higher magnification) as the one indicated by arrows in a. Serial sections c to g show the appearance (e, arrow) and disappearance of a connection between two adjacent surface-connected compartments. Sections were not counterstained. Bars: (a–c) 1 μm; (inset) 0.3 μm.
tensity of plasma membrane staining of macrophages incubated with microcrystalline cholesterol and AcLDL was decreased relative to the level of plasma membrane staining observed in control macrophages (Fig. 1, compare a with b and c). Not only did ruthenium red stain the membranes of compartments that contained the microcrystalline cholesterol and AcLDL, the ruthenium red also stained and outlined the spherical AcLDL and rectangular cholesterol microcrystals in the compartments (making possible the visualization of these lipid particles in thin section).

Extensive compartmentalization occurred in macrophages incubated with microcrystalline cholesterol or AcLDL, and ruthenium red staining indicated these compartments were connected to the extracellular space. However, we did not observe connection of these compartments to the plasma membrane in en face thin sections (i.e., thin sections produced by sectioning macrophage monolayers parallel to the culture surface). This was because connection of these compartments with plasma membranes occurred at the top surface of the macrophage and was visualized in cross sections (i.e., sections perpendicular to the culture surface) (Fig. 2, a and b). Serial cross sections (up to 48 per cell) through the surface-connected compartments (SCC) in 10 cells showed that even what appeared to be separate adjacent compartments, were in fact, interconnected compartments (Fig. 2, c–g). In some sections (Figs. 1 c and 2) compartments could be followed for a long distance through the cytoplasm revealing their elongated and tortuous structure.

Figure 3. Early forming compartment of surface-connected compartments shows invagination of the plasma membrane. Monocyte-macrophage cultures were incubated with 25 μg/ml AcLDL for 3 h (a and c), or with 200 μg/ml microcrystalline cholesterol for 2 h (b). Then, cultures were fixed with glutaraldehyde (without rinsing before fixation), exposed to ruthenium red, and embedded and sectioned perpendicular to the culture surface. Shown are beginning SCC formed by invagination of the plasma membrane. In c, ruthenium red outlined aggregates and chains (arrow) of AcLDL associated with the plasma membrane surface. Sections were not counterstained. Bars: (a) 0.5 μm; (b and c) 1 μm.
Accumulation of Microcrystalline Cholesterol and AcLDL in Lysosomes

A second ultrastructural change within macrophages incubated with microcrystalline cholesterol was the appearance of membrane-bound irregularly shaped organelles with an electron-dense matrix containing embedded cholesterol crystals (Fig. 4a). These irregularly shaped organelles appeared to be derived from fusion of smaller non-crystalline-containing electron-dense organelles (that were also present in control macrophages) with the individual membrane-bound cholesterol crystals (indicated with arrows in Fig. 4a). There was no ruthenium staining of the isolated membrane-bound individual crystals or the crystal-containing electron-dense organelles shown in Fig. 4a. These electron-dense organelles were identified as lysosomes because they contained cathepsin D shown by immunogold labeling (Fig. 4b). Cathepsin D was not present elsewhere in macrophages, including the SCC, which showed that the SCC were not endosomal or lysosomal compartments (Diment et al., 1988).

DiI-labeled AcLDL also accumulated within the lysosomes. DiI-AcLDL was visualized by photoconverting the fluorescent DiI to an insoluble electron-dense product as was described previously (Tabas et al., 1990). Exposure of monocyte-macrophages to UV light produced a darkening of the lysosomes in cells that had been incubated with DiI-AcLDL (Fig. 4c). This method also demonstrated the accumulation of DiI-AcLDL in the SCC. Photoconversion produced a darkening around the edge of the spherical lipoprotein particles within the SCC. There was no photoconversion product in macrophages in areas of the monolayers that were not photoilluminated (Fig. 4d). Also, there was no photoconversion product in macrophages that had not been incubated with DiI-AcLDL, or that had been incubated with unlabeled AcLDL.

We showed previously (Skarlatos et al., 1993a) that macrophages accumulate cholesteryl ester in lipid droplets when these cells are incubated with microcrystalline cholesterol or AcLDL. Although control macrophages contained no cholesteryl ester, they nevertheless contained many lipid droplets that were most prominent in the peripheral cytoplasm.

Examination of cross sections of macrophages at early times (e.g., 2–3 h) after incubation with microcrystalline cholesterol or AcLDL showed that the SCC formed from invaginations of the plasma membrane (Fig. 3a and b). The cell surface openings of these invaginations ranged between 0.2 to 1 \( \mu m \) wide. The invaginations sometimes were and sometimes were not (as in Figs. 2 and 3) associated with microvillous extensions of the macrophages. The membranes of the invaginations and mature SCC did not demonstrate any clathrin-like coating on their cytoplasmic aspect.

**Figure 4.** Accumulation of microcrystalline cholesterol and AcLDL in lysosomes. Monocyte-macrophage cultures were incubated with 400 \( \mu g/ml \) microcrystalline cholesterol for 4 d and processed by routine electron microscopy (a). The central irregular electron-dense inclusion contains many cholesterol crystals. Arrows indicate isolated membrane-bound cholesterol crystals. Gold immunolabeling showed that the electron-dense inclusions with cholesterol crystals contained cathepsin D (b) and thus are lysosomes. Similar irregular electron-dense lysosomal inclusions (but without crystals) were observed in monocyte-macrophages incubated with 25 \( \mu g/ml \) AcLDL for 1 d. After incubation of monocyte-macrophages with DiI-labeled AcLDL, exposure of the monocyte-macrophages to UV light in the presence of diaminobenzidine caused darkening of the lysosomal inclusions (c). No darkening occurred in the absence of UV exposure (d), or when monocyte-macrophages were incubated with unlabeled AcLDL and exposed to UV light (not shown but same as d). a and b were counterstained; c and d were not counterstained. Bars: (a, c, and d) 0.3 \( \mu m \); (b) 0.2 \( \mu m \).
The presence of lipid droplets in control macrophages is consistent with the fact that control macrophages contain triglyceride (Skarlatos et al., 1993b), a lipid in addition to cholesteryl ester, that is stored in droplets. The number of lipid droplets did increase somewhat during cholesterol-enrichment of the macrophages.

Cholesterol Moved from the Surface-connected Compartments into Lysosomes and Lipid Droplets

Macrophages were incubated with [3H]cholesterol (50 µg/ml in microcrystalline form) to compare the early (3 h) and late (48 h) distributions of accumulated [3H]cholesterol in homogenized macrophages fractionated by percoll density gradient centrifugation (Fig. 5, Experiment I). After 3 h of incubation, [3H]cholesterol had accumulated mostly in a cellular compartment with intermediate density on the percoll gradient. The fact that microcrystalline cholesterol was located predominantly within SCC at 3 h indicates that the intermediate density compartment of the percoll gradient corresponded to the SCC. A lesser amount of cholesterol accumulated in a high density lysosomal compartment. Activity of the lysosomal enzyme, β-hexosaminidase, was restricted to this high density compartment. The distribution of [3H]cholesterol after 48 h of incubation showed that the intermediate density SCC contained only slightly more [3H]cholesterol than it did after 3 h of incubation. However, there was a large increase in [3H]cholesterol in the high density lysosomal compartment. In addition, by 48 h, cholesterol had appeared in a low density compartment. Thin layer chromatographic analysis showed the [3H]cholesterol in the low density compartment was greater than 75% esterified, indicating that this was the lipid droplet compartment. [3H]cholesterol in the intermediate density SCC and high density lysosomal compartments was greater than 85% unesterified cholesterol.

The movement of cholesterol between compartments was examined with a pulse-chase experiment. Macrophage cultures were again incubated with [3H]cholesterol for 3 h as in Experiment I. This was followed by a second incubation with unlabelled cholesterol for an additional 48 h to determine the subsequent movement of the [3H]cholesterol that had accumulated during the initial 3 h of incubation. The initial 3-h-accumulation of [3H]cholesterol produced mainly cholesterol-enrichment of the intermediate density compartment similar to that described in Experiment I. One-third of the [3H]cholesterol in the intermediate density compartment left this compartment during the first 24 h of the 48-h chase period. During the 48-h chase period with unlabelled cholesterol, [3H]cholesterol substantially redistributed from the intermediate density compartment into the low and high density compartments (Fig. 5, Experiment II). A similar redistribution of [3H]cholesterol also occurred when the chase medium contained no cholesterol. This showed that the redistribution of [3H]cholesterol did not simply occur by exchange of labeled cholesterol with unlabelled cholesterol in the medium and further exchange of this [3H]cholesterol.
Table I. Effect of Cytochalasin D on Macrophage Cholesterol Accumulation and Esterification Induced by Microcrystalline Cholesterol

| Treatment                        | Cholesterol content |        |        |
|----------------------------------|---------------------|--------|--------|
|                                  | Total               | Unesterified | Esterified |
| 0d                               | 88 ± 9              | 88 ± 9 | 0      |
| 1d + Chol                        | 304 ± 12            | 216 ± 9 | 88 ± 3 |
| 1d + Chol + Cyt D                | 163 ± 7             | 156 ± 5 | 7 ± 2  |
| 1d + Chol; 2d − Chol             | 230 ± 12            | 120 ± 6 | 130 ± 14 |
| 1d + Chol; 2d − Chol + Cyt D     | 265 ± 12            | 119 ± 5 | 146 ± 8 |

Monocyte-macrophage cultures were incubated with 50 μg/ml cholesterol in the presence or absence of cytochalasin D (4 μg/ml) for 1 d. Some cultures that had been incubated with cholesterol for 1 d were rinsed free of exogenous cholesterol. These cultures were then incubated in the absence of cholesterol for an additional 2 d either with or without cytochalasin D. Following incubations, the cholesterol and protein content of cultures were determined as described in Materials and Methods. Values are averages ± SEM of three separate cultures. Chol, cholesterol; cyt D, cytochalasin D; −, without; +, with.

Effect of Cytochalasin D on Accumulation of AcLDL and Microcrystalline Cholesterol

Macrophages were incubated with AcLDL and microcrystalline cholesterol in the presence of cytochalasin D, a known inhibitor of microfilament function and phagocytosis (Cooper, 1987). Cytochalasin D prevented accumulation of AcLDL-derived cholesterol, and partially blocked accumulation of cholesterol derived from microcrystalline cholesterol (Tables I and II). Even though macrophages accumulated a substantial (although reduced) amount of microcrystalline cholesterol in the presence of cytochalasin D (Table I), cytochalasin D blocked almost completely esterification of this cholesterol. Incubation of macrophages with fluorescent NBD-cholesterol in the presence of cytochalasin D demonstrated that the block in esterification of cholesterol was associated with a failure of macrophages to sequester microcrystalline cholesterol in the perinuclear region (Fig. 6). Rather, in the presence of cytochalasin D, some cholesterol crystals adhered to the surface of the macrophages, but none were present within macrophages. Thus, these surface-bound cholesterol crystals accounted for the increase in the cholesterol content of macrophages that occurred when internalization of microcrystalline cholesterol was blocked by cytochalasin D (Table I). However, this surface-bound cholesterol was not available for esterification.

Table II. Effect of Cytochalasin D on Macrophage Cholesterol Accumulation Induced by AcLDL

| Duration of incubation | Cholesterol content |        |        |
|------------------------|---------------------|--------|--------|
|                        | nmol/mg protein     |        |        |
|                        | Without cytochalasin D | With cytochalasin D |
| 0d                     | 67 ± 5              | −      |
| 1d                     | 137 ± 10            | 76 ± 11 |
| 2d                     | 154 ± 4             | 71 ± 2  |

Monocyte-macrophage cultures were incubated with 100 μg/ml AcLDL in the presence or absence of cytochalasin D (4 μg/ml) for the indicated number of days. Following incubations, the cholesterol and protein content of cultures were determined as described in the Materials and Methods. Values are averages ± SEM of three separate cultures.

Cytochalasin D did not block esterification of cholesterol that was already sequestered in macrophages. This was shown by allowing macrophages to accumulate cholesterol for 24 h, rinsing the cultures free of exogenous cholesterol, and then comparing the amount of cholesterol esterified during a subsequent 48-h incubation in medium without or with cytochalasin D. Similar amounts of additional cholesterol (~50 nmol/mg cell protein) were esterified in both conditions during the 48-h post-incubation period (Table I). The results taken together, demonstrate that cytochalasin D blocked intracellular sequestration of cholesterol and that sequestration was a necessary step before cholesterol es-

Figure 6. Effect of cytochalasin D on monocyte-macrophage uptake of fluorescent NBD-cholesterol. Monocyte-macrophage cultures were incubated with 50 μg/ml NBD-cholesterol for 24 h in the absence (a and b) or presence (c and d) of cytochalasin D (4 μg/ml). Cytochalasin D blocked the sequestration of NBD-cholesterol crystals. However, NBD-cholesterol crystals were adherent to the macrophage surface in the presence of cytochalasin D. a and c are fluorescence micrographs of macrophages, and b and d are respective phase micrographs of the same cells. Bar, 20 μm.
Table III. Cholesterol Efflux from Macrophages After Incubation with Microcrystalline Cholesterol

| Treatment | Total nmol/mg protein | Unesterified nmol/mg protein | Esterified nmol/mg protein |
|-----------|-----------------------|------------------------------|---------------------------|
| 0d        | 92 ± 3                | 91 ± 3                       | 1                         |
| 1d + chol | 675 ± 22              | 585 ± 32                     | 91 ± 22                   |
| 1d + chol; 4d - chol | 236 ± 5              | 142 ± 4                      | 94 ± 6                    |

Monocyte-macrophage cultures were incubated with RPMI 1640 medium with 400 μg/ml microcrystalline cholesterol for 1 d. Cultures were then rinsed and incubated in RPMI 1640 medium without microcrystalline cholesterol for an additional 4 d. At the indicated times, cholesterol and protein contents of cultures were determined as described in the Materials and Methods. Values are averages ± SEM of three separate cultures. chol, cholesterol; −, without; +, with.

Cholesterol efflux was determined as described in the Materials and Methods. Values are averages ± SEM of three separate cultures. Chol, cholesterol; −, without; +, with.

terification could occur. Microtubules did not appear to function in uptake or esterification of cholesterol. Colchicine (50 μg/ml), an inhibitor of microtubule function, was without effect on cholesterol accumulation or esterification.

**Nature of AcLDL that Entered Surface-connected Compartments**

AcLDL that entered the surface-connected compartments appeared to be aggregates of individual spherical lipoprotein particles (some of which were up to 5 x the 22-nm size of AcLDL [Vanderyse, 1992]) (Fig. 2 b). These aggregates often appeared in chains that were loosely associated with the macrophage cell surface at 3 h of incubation of AcLDL with the macrophages (Fig. 3 c). The aggregates formed during incubation of AcLDL with the macrophages, because filtration (0.45 μm pore-size) of AcLDL before its addition to the macrophages did not eliminate the occurrence of the aggregates, and no aggregates were seen in negatively stained preparations of AcLDL that were added to the culture medium. Also, filtration of AcLDL did not change the degree of cholesterol accumulation induced by the AcLDL. The cholesterol content of macrophages incubated for 2 d with filtered AcLDL (100 μg/ml) was 259 ± 35 nmol compared to 223 ± 4 nmol for unfiltered AcLDL. The aggregates of AcLDL were not induced by the ruthenium red stain because similar lipoprotein aggregates were present when Dil-AcLDL was visualized by photoconversion.

**Reversal of Surface-connected Compartments**

We next determined what happened to the SCC when microcrystalline cholesterol was removed from the medium after SCC were first induced. Macrophages were incubated one day with microcrystalline cholesterol (400 μg/ml) and then incubated an additional four days in cholesterol-free (and serum-free) medium. During this period, macrophages lost 90% of their accumulated unesterified cholesterol (443 nmol/mg cell protein) but lost none of their accumulated esterified cholesterol (Table III). In efflux experiments after incubation of macrophages with cholesterol for one day), macrophages sometimes showed an increase in cholesteryl ester content during the efflux period, and sometimes showed no change in the cholesteryl ester content (as in Table III). It was not clear what led to varying cholesteryl ester synthesis during cholesterol efflux, and this response did not correlate with the level of cholesterol enrichment of the macrophages. At the same time that macrophages lost their accumulated unesterified cholesterol during cholesterol efflux, the SCC and their contained microcrystalline cholesterol disappeared from the macrophages (Fig. 7). Also, as macrophages lost their accumulated unesterified cholesterol, ruthenium red staining of the plasma membrane increased in intensity (more similar to control macrophages that had not been incubated with microcrystalline cholesterol).

**Demonstration of Apolipoprotein E in the Surface-connected Compartments**

Because of the importance of apolipoprotein E in lipid particle trafficking (Tabas et al., 1991) and cholesterol efflux from macrophages (Kruth et al., 1994), we examined whether apolipoprotein E (which the macrophages are known to synthesize [Basu et al., 1981]) was present in the SCC. Immunogold labeling of apolipoprotein E showed this protein was present in the SCC and within some cytoplasmic granules (the granules were also present in control macrophages) (Fig. 8). The granules that contained the apolipoprotein E did not contain microcrystalline cholesterol and thus were not the lysosomes described above. There was no gold labeling with control antibodies, and preincubation of the mouse anti-apolipoprotein E monoclonal antibody with purified apolipoprotein E blocked labeling of apolipoprotein E in sections.

**Discussion**

We have shown that exposure of human monocyte-macrophages to microcrystalline cholesterol or AcLDL results in extensive compartmentalization of the macrophage cytoplasm. Compartmentalization occurred in the peripheral cytoplasm as well as deep within the cell in the perinuclear region. Unexpectedly, the compartments, including those that appeared deep within the cell, were in continuity with and opened to the extracellular space. The compartments were often elongated and tortuous, and adjacent compartments were often connected to one another. Compartmentalization was not specifically related to uptake of cholesterol-containing particles as 0.2-μm latex beads also induced similar SCC (unpublished data).

The SCC appear to form through focal invaginations of the plasma membrane induced by cholesterol microcrystals or aggregates of AcLDL. The compartments were not just invaginations from ruffled portions of the plasma membrane. Openings of many compartments arose from smooth rather than ruffled segments of the plasma membrane. Also, the compartments did not contain cathepsin D which differentiated these compartments from endosomes and lysosomes (Diment et al., 1988). Thus, human monocyte-macrophages
Figure 7. Reversal of surface-connected compartments upon removal of microcrystalline cholesterol from the medium. Monocyte-macrophage cultures were incubated with 200 μg/ml microcrystalline cholesterol for 1 (a) or 5 d (b). Another culture was incubated with 200 μg/ml microcrystalline cholesterol for 1 d followed by incubation in RPMI 1640 medium without microcrystalline cholesterol for an additional 4 d (c). All cultures were fixed in glutaraldehyde, exposed to ruthenium red to delineate the SCC, and then further processed for electron microscopy. The photomicrographs show the disappearance of SCC during the 4 d after incubation in cholesterol-free medium. Sections were not counterstained. Bar, 1 μm.

Figure 8. Immunogold labeling of apolipoprotein E in macrophages. Monocyte-macrophages were incubated with 200 μg/ml microcrystalline cholesterol for 1 d. Monolayers were fixed, scraped from culture dishes, and embedded in LR White resin for post-embedding gold immunolabeling. Apolipoprotein E was labeled in thin sections with 10 μg/ml of a purified mouse anti-apolipoprotein E monoclonal antibody. The anti-apolipoprotein E monoclonal antibody was then detected with purified goat anti-mouse Ig-colloidal gold (10 nm). Shown in a are three small granules (arrows) heavily labeled with gold. The granules were present in control and cholesterol-enriched monocyte-macrophages. The inset shows one such labeled granule at higher magnification. SCC (b) was the only other structure in monocyte-macrophages that showed immunogold labeling of apolipoprotein E. Neither the granules or SCC labeled when a control mouse monoclonal antibody was substituted for the mouse anti-apolipoprotein E antibody, or when the mouse anti-apolipoprotein E antibody was first absorbed with purified apolipoprotein E. Bar: (a) 0.25 μm; (b) 0.35 μm. The 10-nm gold particles can be used as a size marker for the inset.

process AcLDL and cholesterol microcrystals by an unusual pathway that resembles phagocytosis. However, rather than forming vacuoles which rapidly pinch off from the plasma membrane as occurs during phagocytosis, some AcLDL and cholesterol microcrystals remained sequestered within the SCC (one day for AcLDL and up to 4 d for the cholesterol microcrystals). Formation of the SCC appears to result from a reorganization of existing cell membranes as there was no increase in the phospholipid content of the macrophages as SCC developed.

A related phenomenon has been described in the uptake of large particulates by human blood platelets. White (1972)
has shown that latex particles enter the open canicular system of platelets, a system of channels that open to the extracellular space. These channels can accumulate latex particles and at the same time remain open to the extracellular space. The channels in platelets preexist, while the SCC were induced by the microcrystalline cholesterol and AcLDL that were added to the culture medium. Recently, Myers et al. (1993) reported on sequestration of β-VLDL in surface-connected tubules in mouse peritoneal macrophages. The surface-connected tubules in the mouse peritoneal macrophages and the SCC in the human monocyte-macrophages are likely analogous structures. However, the surface-connected tubules in mouse peritoneal macrophages do not appear to be as extensive as the SCC in human monocyte-macrophages. Also, some of the surface-connected tubules in mouse peritoneal macrophages showed coated regions, whereas we found no coated membrane regions along the SCC. However, it is possible that coated membrane regions were present at an earlier time but were lost during formation of the SCC.

An interesting finding was that aggregates of AcLDL formed during incubation with the macrophages and that these aggregates were sequestered. Previous studies suggest that aggregation of lipoproteins greatly facilitates their uptake by macrophages (Koo et al., 1988; Suits et al., 1989; Tertov et al., 1989; Hoff and Cole, 1991; Xu and Tabas, 1991; Hoff et al., 1992). A study by Tertov et al. (1992) showed that many other types of modified low density lipoproteins aggregate during incubation with cultured cells, and that formation of these aggregates is necessary for cellular cholesterol accumulation to occur. Our results suggest that aggregate may be involved in uptake of AcLDL by human monocyte-macrophages.

A decrease in ruthenium red staining of the plasma membranes of macrophages has been observed previously during receptor ligand interactions (Richards and Douglas, 1983). We observed a similar decrease in staining when macrophages were incubated with the AcLDL and microcrystalline cholesterol. The decrease in staining has been attributed to a decrease in thickness of the polyanionic, extracellular glyocalyx. Although the mechanism for a reduction in the extracellular glyocalyx has not been established, it was shown that treatment of cells with trypsin or neuraminidase decreases the cell glyocalyx similar to the decrease that occurs during receptor ligand interactions. Interestingly, elevated lipoprotein levels in vivo decreases the glyocalyx of endothelial cells, and in swine, preferentially those endothelial cells covering developing atherosclerotic lesions (Gerity, 1977; Lewis, 1982).

Pulse-chase studies showed that cholesterol moved through the SCC on its way into lysosomes (where the cholesterol remained unesterified) and into lipid droplets (where the cholesterol was stored as cholesteryl ester). Cytochalasin D, an inhibitor of microfilaments (Cooper, 1987), blocked internal sequestration of the AcLDL and cholesterol microcrystals. Even though cholesterol microcrystals bound to the surface of macrophages in the presence of cytochalasin D, the bound cholesterol did not reach sites in the cell where cholesterol could be esterified. This confirmed that cholesterol only became available for esterification after sequestration into the SCC. Once cholesterol was internally sequestered in SCC, transport to sites of esterification was no longer susceptible to inhibition by cytochalasin D.

Apolipoprotein E in the SCC may be important in the trafficking and solubilization of lipid particles taken into the SCC. Tabas et al. (1991) previously showed that the apolipoprotein E content of lipoprotein particles influenced the trafficking of lipoproteins in the macrophages, and increased the potential of the lipoproteins to stimulate cellular cholesterol esterification. In other work, we found that human monocyte-macrophages progressively solubilized some microcrystalline cholesterol (up to 50 nmol/mg cell protein/4 d) in discoidal lipoprotein particles containing apolipoprotein E during and following incubation of the macrophages with microcrystalline cholesterol (Kruth et al., 1994). Thus, the presence of apolipoprotein E in the SCC possibly contributes to this solubilization process. How apolipoprotein E entered the SCC (e.g., directly from the apolipoprotein E-containing cytoplasmic granules or indirectly after macrophages secrete apolipoprotein E into the medium) remains to be determined. Werb et al. (1989) previously localized apolipoprotein E in basal cytoplasmic compartments induced in mouse peritoneal macrophages undergoing frustrated phagocytosis of immune complexes attached to the culture surface. In that study, apolipoprotein E was also visualized in cytoplasmic granules that these investigators believed emptied into the basal compartments.

The full significance of sequestration of cholesterol microcrystals and AcLDL into compartments that maintain a connection to the extracellular space remains to be determined. Similar to classic phagocytosis, macrophage sequestration of lipid particles provides a means to remove these substances from the extracellular space. However, unlike phagocytosis, in the case of microcrystalline cholesterol, SCC not only functioned as a reservoir of cholesterol to be converted to cholesteryl ester and stored in the macrophage, but also SCC stored excess microcrystalline cholesterol that was released from the cells when extracellular levels of microcrystalline cholesterol were reduced. The potential usefulness of this type of temporary storage is better understood when one considers that macrophages are mobile cells that enter and leave tissues (Gerity, 1981). Possibly discharge of cholesterol from the SCC occurs after a macrophage leaves a tissue site (such as an atherosclerotic lesion) and reenters the blood stream. It remains to be determined whether similar release of lipoprotein particles from the SCC can occur when their uptake exceeds the capacity of macrophages to degrade them.

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The Journal of Cell Biology, Volume 129, 1995
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Kruth et al. Macrophage Sequestration of Lipid Particles 145