Sequestration of aggregated LDL by macrophages studied with freeze-etch electron microscopy

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Abstract **The detailed morphology of macrophages involved in the uptake and intracellular processing of low density lipoprotein (LDL) and, ultimately, formation of macrophage-derived foam cells of atherosclerotic lesions has long fascinated investigators. This study examined localization of LDL in subcellular compartments of macrophage-derived intimal foam cells in cardiac valves isolated from rabbits by diet-induced hypercholesterolemia and, as an in vitro model of formation of foam cells, in cultured human monocyte-macrophages incubated for 2–120 h with aggregated LDL produced by vortexing or phospholipase C lipolysis. The quasi-three-dimensional morphology of macrophages involved in endocytosis was preserved by ultrarapid freezing and freeze-etch microscopy in conjunction with thin-section electron microscopy. This approach produced unique images of subcellular compartments in human monocyte-macrophages involved in the uptake and processing of aggregated LDL with a clarity not previously reported. Three-dimensional ultrastructural analyses revealed a complex network of coated and uncoated vesicles, surface-connected saclike compartments, and endosomal/lysosomal compartments including the labyrinth of vesicular/tubular lysosomes all enmeshed in the microtubular, microfilament cytoskeletal network. These dynamic views of subcellular structures at the high resolution of the electron microscope provide an additional framework to better understand how lipoprotein particles are transported into, and processed within, macrophages during foam cell formation in atherogenesis.**—Haberland, M. E., G. Mottino, M. Le, and J. S. Frank. **Sequestration of aggregated LDL by macrophages studied with freeze-etch electron microscopy.** ***J. Lipid Res.*** **2001.** 42: **605–619.**

Supplementary key words LDL-gold • surface-connected compartments • surface tubules for entry into macrophages • CURL • atherosclerosis

The appearance of macrophage-derived foam cells in the arterial wall is an early morphological hallmark of the atherosclerotic lesion (1–3). These arterial cells are laden with lipoprotein-derived cholesterol, chiefly in the form of intracellular droplets of cholesteryl ester, and it is widely accepted that the internalization and intracellular processing of lipoproteins by specialized pathways account for the massive intracellular accumulation of lipid (4). Modifications of low density lipoprotein (LDL) such as oxidation or aggregation have been shown to enhance LDL uptake by macrophages and produce rapid intracellular cholesterol accumulation and foam cell formation (reviewed in 5, 6). Aggregated LDL presented to macrophages in vitro is taken up and degraded in endosome/lysosomal compartments and accumulates within the cell as cytoplasmic droplets of cholesteryl esters (7, 8). In vivo, the oxidation and subsequent aggregation of LDL are thought to occur primarily in the subendothelial matrix. Several studies have suggested that the retention and aggregation of arterial LDL occurs in the subendothelial matrix as a prelesional event even before monocytes enter the artery wall (9–11).

The uptake of altered lipoproteins by macrophages is a complex process that may involve several pathways. Receptor-mediated endocytosis and phagocytosis (4, 7, 8) are two well-described routes. Unique endocytic pathways consisting of an extensive network of surface-connected tubules have been described in cultured macrophages. These compartments sequestered aggregated LDL in human monocyte-macrophages (12) and β-very low density lipoprotein in mouse peritoneal macrophages (13). Kruth and colleagues (12) demonstrated continuity of surface-connected compartments with the cell surface of human monocyte-macrophages by thick-section electron microscopy after labeling with ruthenium red. Zha and colleagues (13) provided a more three-dimensional view of surface tubules for entry into macrophages in studies with confocal microscopy and high voltage electron microscopy. It appears that these unique endocytic pathways are important for the uptake and processing of lipoproteins in macrophages. The detailed morphology of endocytic structures and their relationship to other intracellular structures such as the cytoskeleton are still incompletely understood. This is due, in part, to the limitations of light and conventional electron microscopy in acquiring three-dimensional high resolution images.

In the current study we exposed cultured human mono-

Abbreviations: LDL, low density lipoprotein; PLC-LDL, LDL enzymatically treated with phospholipase C; VX-LDL, vortexed LDL.

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cyte-macrophages to aggregated LDL produced by vortexing (7) and by phospholipase C (PLC) lipolysis (8, 14). The quasi-three-dimensional morphology of the macrophages involved in endocytosis was preserved by ultrarapid freezing and freeze-etch microscopy in conjunction with thin-section electron microscopy. This approach produced unique images of the saclike compartments in association with the cell surface and endosomal/lysosomal compartments, including the labyrinth of vesicular/tubular compartments involved in the uptake of aggregated lipoproteins, with a clarity not previously reported.

MATERIALS AND METHODS

Materials

PLC (grade I) from *Bacillus cereus* was obtained from Boehringer Mannheim (Indianapolis, IN), 5-nm colloidal gold was from Ted Pella (Redding, CA), and Sephadex G-50 resin was from Pharmacia (Piscataway, NJ). Falcon cell culture inserts with 0.45-μm pore size polyethylene terephthalate membranes and companion 24-well cluster plates were purchased from Fisher Scientific (Pittsburgh, PA); Centriprep-30 concentration units were from Amicon (Beverly, MA) and Acrodisc syringe filter units, 0.2-μm pore size, were from Gelman Sciences (Ann Arbor, MI). Tissue culture reagents were purchased from BioWhittaker (Walkersville, MD) and GIBCO-BRL (Gaithersburg, MD). All other reagents of the highest quality were obtained from Sigma (St. Louis, MO) and Fisher Scientific.

Human subjects

Normal human subjects at the University of California, Los Angeles (UCLA) were recruited from the staff and student body as donors for blood monocytes and LDL. No one received drugs that might have affected serum cholesterol levels or lipid metabolism. All had normal hemocrits, leukocyte and differential counts, serum cholesterol, and triglycerides. Informed consent was obtained in writing from each subject. Institutional approval was also obtained.

Lipoprotein isolation

Human LDL (d 1.019–1.063 g per ml) was isolated from the plasma of individual, fasted human subjects by ultracentrifugation (15) and dialyzed against 0.01 M sodium phosphate, pH 7.4, containing 0.15 M sodium chloride and 0.01% ethylenediaminetetraacetic acid (EDTA). Lipoprotein solutions were sterile filtered (0.2-μm pore size) on completion of dialysis and stored at 4–5°C in the presence of penicillin (100 units/ml) and streptomycin (100 μg/ml). Protein concentrations were determined by the method of Lowry et al. (16).

Vortexed LDL

Aggregated LDL was generated as described by Khoo et al. (7) just before addition to cultured cells. Sterile LDL (1 mg of protein per ml) was subjected to high speed vortexing for 1 min. The preparation was immediately diluted to 0.5 mg of protein per ml with medium B [Iscove's modified Dulbecco's medium supplemented with 2 mM glutamine, insulin (8 μg/ml), penicillin (100 units/ml), streptomycin (100 μg/ml), and Fungizone (amphotericin B, 0.25 μg/ml)]-containing autologous serum.

Colloidal gold labeling of lipoprotein

LDL (4.8 mg of protein) was labeled with 5-nm colloidal gold as previously described (17, 18). The basic methodology of colloidal gold labeling and preservation of the biological characteristics of the ligand is now firmly established (18). The concentration of LDL at which optimal labeling occurred without significant gold remaining (≤1% free colloidal gold) was determined as previously described (17). Colloidal gold-labeled LDL was concentrated by centrifugation in Centriprep-30 filter units to 1 mg of LDL protein per ml, sterilized by 0.2-μm pore size filtration, and stored at 4°C under argon for fewer than 5 days.

Modifications of colloidal gold-labeled LDL

PLC-hydrolyzed LDL (PLC-LDL) was generated as described by Heinecke et al. (14) by incubation of colloidal gold-labeled LDL (100 μg of protein per ml) with PLC (1 enzyme unit/ml) for 1 h at 37°C. The enzymatically treated LDL was reisolated by chromatography on Sephadex G-50 (0.9 × 10 cm) in 50 mM Tris-HCl buffer, pH 7.4, containing 0.01% EDTA, concentrated by centrifugation in Centriprep-30 filter concentration units to 1 mg of LDL protein per ml, sterilized by 0.2-μm pore size filtration, and stored at 4°C under argon.

Aliquots of all preparations were examined by electron microscopy after negative staining with 2% phosphotungstic acid to confirm retention of colloidal gold label and validate lipoprotein morphology.

Cardiac valves

New Zealand White rabbits (2.5 kg, 8 weeks) were maintained for 14 days on a chow diet containing 2% cholesterol (w/w) in soybean oil (10%, w/w). All animal protocols conformed to institutional guidelines on care and handling of animals. Cardiac valves were isolated and prepared for incubation as previously described (17). The same procedures were used in this study and are briefly given here. The leaflets of each valve remained intact by a small ring of connective tissue. The valves were rinsed in phosphate-buffered saline (PBS)-$NaHCO_3$ buffer (104 mM Na_2HPO_4, 32 mM KH_2PO_4, 125 mM NaCl, 12 mM $NaHCO_3$, pH 7.2) and subsequently incubated with either LDL-gold conjugate (125 μg/ml) in PBS/$NaHCO_3$ buffer supplemented with human lipoprotein-deficient serum (4.5 mg/ml) for 4 h or with LDL (300 μg/ml) for 2 h in a humidified, 5% CO_2 incubator. Tissue pieces were cut from the intact valves and either immediately processed for thin-section microscopy or ultrarapidly frozen for freeze etching.

Human monocyte isolation and culture

Five hundred milliliters of blood was taken after an overnight fast, and the monocytes were separated from 300 ml of venous blood by a modification of the Recalde method (19). The cells were classified and their viability was determined as described. Autologous serum for cell culture was prepared from the remaining 200 ml of blood. Human monocytes were suspended in medium B containing 30% autologous human serum. Cell suspensions containing 2.5 × 10^5 monocytes were transferred to cell culture inserts (250 μl), and the inserts were placed in wells (750 μl) containing medium B supplemented with 30% autologous serum. The cells were maintained for 7–11 days at 37°C in a humidified, 5% CO_2 incubator. The media were aspirated and replaced with fresh medium of the same composition twice weekly. Because of the medium changes and the washes, the cells were 99% monocyte-macrophages before initiation of each experiment. More than 95% of the cells were viable at the conclusion of the experiment.

For incubation with lipoproteins, human monocyte-macrophages cultured on the porous inserts were washed three times with 1-ml volumes of Dulbecco's modified Eagle's medium containing 10 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid. Lipoproteins in medium B supplemented with 5–10% autologous serum were added to the inserts. The units were placed

in fresh wells containing medium B supplemented with 5–10% autologous serum, and the cells were returned to the incubator for the indicated times. For incubations extending beyond 72 h, the media were aspirated and replaced once with fresh medium of the same composition.

At the time of ultrarapid freezing the membrane inserts with the adherent, cultured cells were carefully and rapidly removed from the plastic housing units and placed on the freezing head. Freezing occurred within 30 s of removal of the inserts from the chambers.

Freeze-fracture and thin section electron microscopy

The tissue was fractured on a Balzers 301 freeze-fracture apparatus (Bal-Tec, Balzers, Liechtenstein). The macrophages still adherent to the filters and the isolated cardiac valves were fractured superficially (<10 μm) to ensure good freezing. The

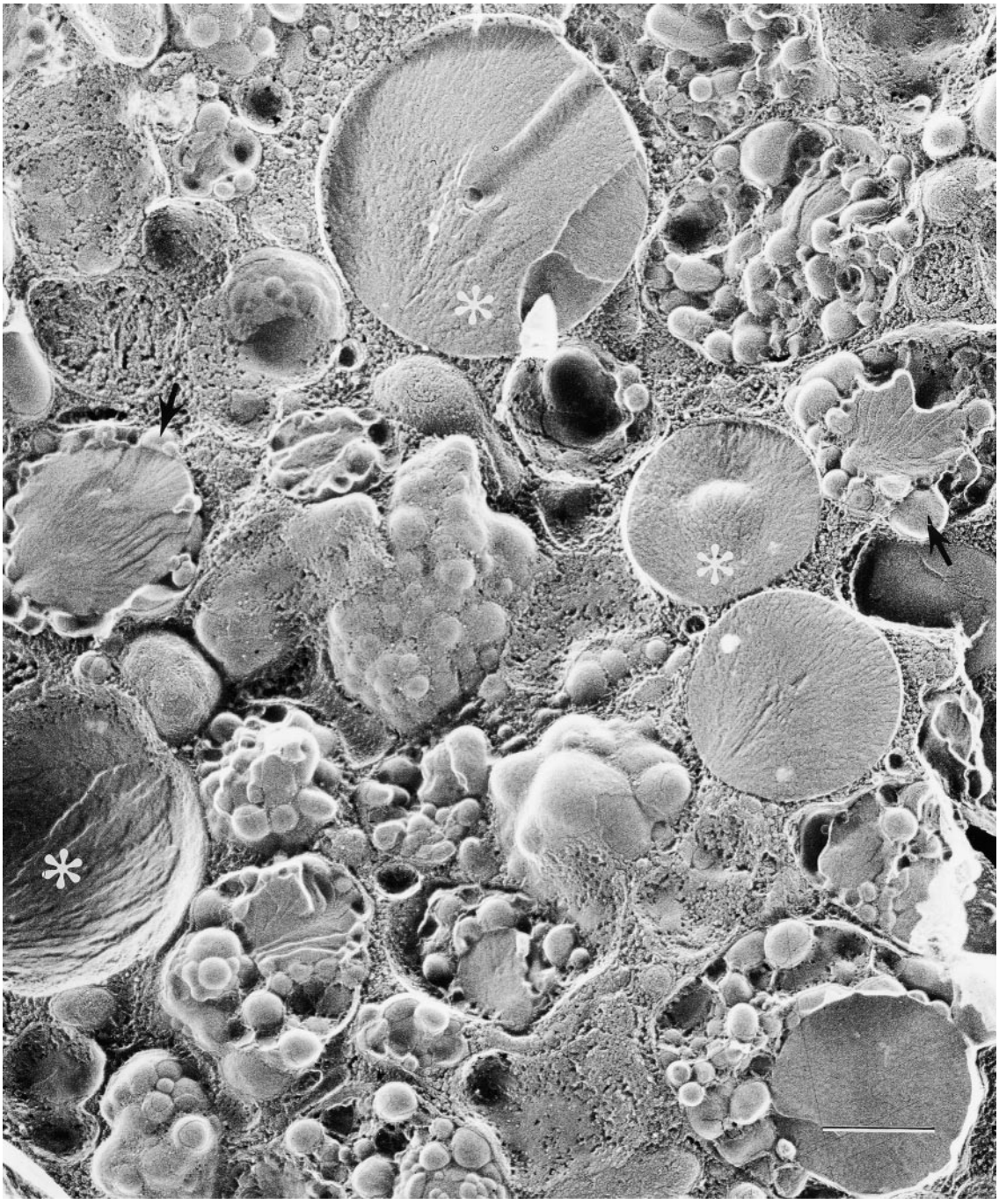


Fig. 1. Freeze-etch electron micrograph of a portion of a macrophage foam cell in the intima of a rabbit cardiac valve. The rabbit had been maintained on a high fat diet for 2 weeks to induce the development of foam cells in the intima. The cytoplasm is filled with lipid in various intracellular compartments. Large lipid droplets with the onion skin configuration typical of cholesteryl esters are also present (asterisk). Many membrane-bound compartments contain clusters of various sized lipid particles. The arrows indicate compartments where the aggregated lipid particles appear in close association with large lipid droplets similar in morphology to cholesteryl esters. Original magnification: ×33,048.

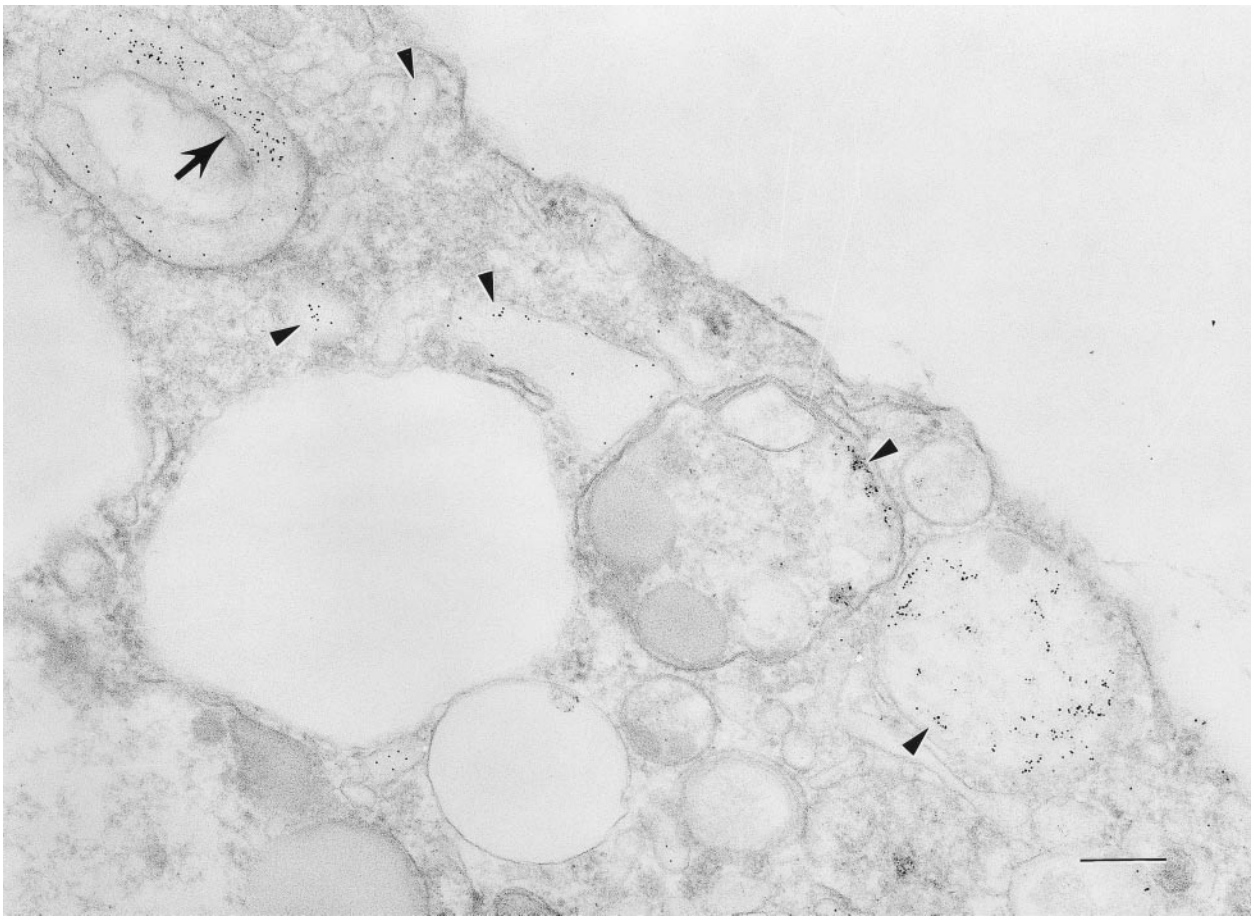


Fig. 2. Thin-section electron micrograph of a portion of a macrophage in the intima of a rabbit cardiac valve that had been isolated and incubated for 4 h with gold-tagged low density lipoprotein (LDL). The rabbit had been fed a high fat diet for 2 weeks before sacrifice, resulting in the development of foam cells in the intima. The localization of the LDL-gold in the various compartments (including endosomal/lysosomal-like vesicles, multivesicular bodies, and in small tubules that appear to connect to the cell surface) can be seen (arrowheads). LDL-gold has also entered a large tubular compartment in the macrophage (arrow). Original magnification: ×53,820.

cells were fractured at −150°C under a vacuum of 1×10^{-7} mmHg.

For deep-etching, the specimen stage was warmed to −110°C and maintained at this temperature for 3 min, followed by 3 min at −100°C and 2 min at −95°C. Rotary shadowing and replica formation were performed as previously described (17, 20). The tissue was digested with household bleach, rinsed in distilled water, and picked up on Formvar-coated grids. A total of 150 replicas from the cultured macrophages, and 50 replicas from the cardiac valves, were examined. Each replica provides ~500 μm^2 of area for examination.

For thin-section microscopy, the cells were fixed in glutaraldehyde followed by OsO_4. The cells were dehydrated in graded concentrations of ethanol and embedded in epoxy resin for ultrathin sectioning. Sections were stained with uranyl acetate and lead citrate and observed with a JEOL (Tokyo, Japan) 100CX electron microscope.

RESULTS

Foam cell ultrastructure in vivo

Cardiac valves are composed chiefly of extracellular matrix surrounded by an endothelial monolayer. In rabbits with diet-induced hypercholesterolemia, the valves develop the same massive accumulation of lipids and macrophage-derived foam cells as found in the aorta (21, 22). Cardiac valves were isolated from rabbits maintained for 14 days on hypercholesterolemic chow and were prepared for ultrastructural analyses as described in Materials and Methods. **Figure 1** shows a typical freeze-etch image of a macrophage-derived foam cell from the intima of a cardiac valve. Ultrarapidly frozen, freeze-etched images of foam cells produced quasi-three-dimensional images that were complex. The structural detail of the cell interior clearly showed lipid inclusions in two major compartments that were typical of foam cells. These compartments included a large, nonmembrane-bound lipid droplet with an "onion skin" configuration typical of cholesteryl esters and a membrane-delimited endosomal/lysosomal compartment that contained aggregates of lipid particles of varying sizes. When the fracture plane passed through the lumen of the membrane-delimited compartment, the lipid particles appeared to be fusing to form larger particles. In some compartments both clusters of lipid particles and the onion skin configuration of cholesteryl esters could be seen.

We have previously demonstrated that incubation of cardiac valves for 4 h with LDL-gold conjugate produced localization of the tagged LDL within the valvular intima (17). Thin-section electron micrographs of cardiac valves from cholesterol-fed rabbits showed LDL-gold particles within multivesicular bodies in the cytoplasm of the endothelial cells and associated with collagen filaments within the subendothelial matrix. The present study examined whether macrophage-derived foam cells present in the cardiac valves also sequestered the LDL-gold conjugate after the 4-h incubation period. Conventional thin-section electron microscopy showed LDL-gold particles present on the macrophage cell surface, and in several compartments within the cytoplasm including membrane-limited vacuoles filled with lipid, in both coated and uncoated endocytic vesicles, and in tubular channels (**Fig. 2**). The experiments with isolated cardiac valves together with previous work (17) demonstrate that during the 4-h incubation period the LDL-gold conjugate was transported across the endothelium into the matrix and sequestered into intracellular compartments within macrophages present in the intima.

Aggregated LDL uptake into cultured macrophages

Routine examination of the early stages of foam cell formation in cardiac valves is limited by several factors. These include variability in individual animal responses to hypercholesterolemic diet and consequently a variable density of macrophage-derived foam cells in the matrix. Moreover, incubations of isolated cardiac valves for more than 4 h resulted in tissue deterioration and further limited flexibility in experimental design.

Cultured macrophages allow greater flexibility over experimental conditions than isolated cardiac valves. Cultured cells are also ideal for freeze-etch studies because the cells of interest are concentrated into a single layer, which increases the likelihood of optimal fracture planes and also enhances the quality of freezing. We turned to the generation of foam cells in culture for ultrastructural analyses, and focused on production of aggregated LDL containing lipid particles comparable in size (23–100 nm in diameter) to those observed in arterial intima and valvular intima of cholesterol-fed rabbits (10, 17, 20). A number of models of LDL fusion and aggregation have been described (23). Of these, vortexed LDL (VX-LDL) (7, 24) (25–58 nm in diameter) and PLC-LDL (8, 14) (23–35 nm in diameter) met the selected ultrastructural criteria.

In a series of experiments, human monocyte-macrophages maintained in culture for 11 days were incubated with high concentrations (500 μg/ml) of either VX-LDL

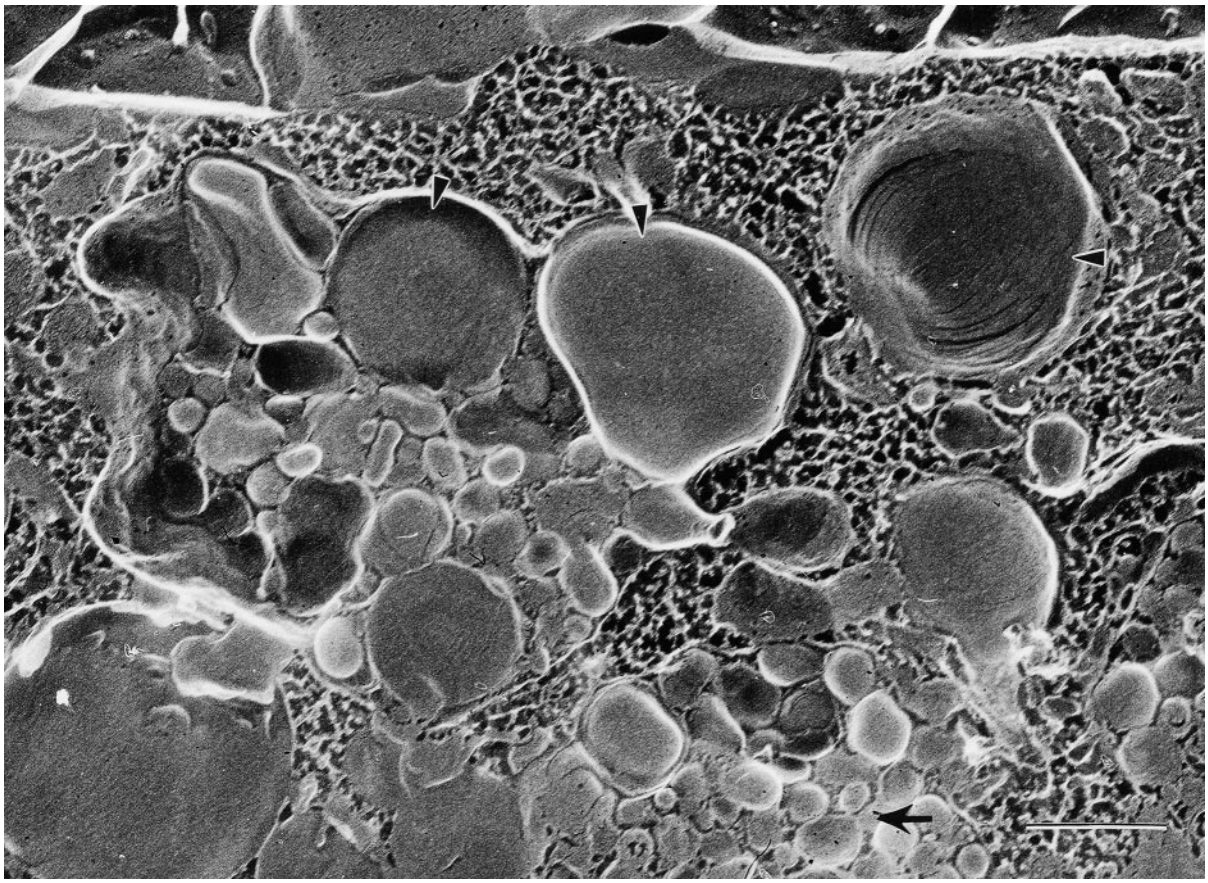


Fig. 3. Freeze-etch electron micrograph of a human monocyte-macrophage incubated for 120 h with vortexed LDL (VX-LDL). Cells were maintained in culture for 11 days, and incubated for 120 h with VX-LDL (500 μg/ml) as described in Materials and Methods. Intracellular compartments contain clusters of lipid similar to those seen in foam cells in vivo (arrow). Also present in the cytoplasm are large lipid droplets with the onion skin configuration of cholesteryl esters (arrowheads). Original magnification: ×35,245.

or PLC-LDL for periods of time ranging from 2 to 120 h. Matched sets of cells incubated with monodisperse, native LDL were included for comparison. None of the monocyte-macrophages treated with native LDL generated foam cells in culture (data not shown).

When observed by freeze-etch microscopy, monocyte-macrophages incubated with VX-LDL for 120 h contained numerous cytoplasmic, membrane-limited compartments filled with clusters of lipid-like particles, typical of aggregated LDL seen in foam cells in the aorta or in cardiac valves (10, 17, 20). Vacuoles laden with lipid in various stages of degradation, and cytoplasmic inclusions that lacked a bilayer membrane and contained the onion skin configuration of cholesteryl ester lipid droplets, were present (**Fig. 3**). The similarity in ultrastructure between these foam cells generated in culture and the in vivo foam cells was striking (compare Figs. 1 and 3), and thus supports the use of cultured macrophages as a model to study LDL sequestration and foam cell formation.

When human monocyte-macrophages were incubated with VX-LDL for 12 h, freeze-etch microscopy of the cells revealed extensive compartments, close to the cell surface, that were filled with large clusters of lipid particles (**Fig. 4A**). These compartments were saclike structures. In fractures through the lumen of the sac, clusters of variously sized lipid particles were readily visible (Fig. 4A). Most of the lipid particles had diameters between 25 and 58 nm. Each lipid aggregate contained some larger particles with diameters between 98 and 145 nm. This is not surprising, because VX-LDL is aggregated and prone to fusion into larger particles (24). In advantageous fracture planes it was apparent that these sacs were connected to the cell surface via a neck or tubule (Fig. 4B).

To confirm that the particles present in the surface-connected compartments were derived from the aggregated LDL presented to the cell during the experimental incubation, LDL was tagged with gold to identify its origin. Unfortunately, vortexing stripped the gold label from the lipoprotein. PLC hydrolysis of gold-conjugated LDL was used as an alternative approach to generate aggregated, colloidal gold-tagged LDL.

After exposure to PLC-LDL-gold for 2 h, or the more typical 12-h incubations, the cultured macrophages contained saclike compartments in their cytoplasm filled with aggregated lipoprotein particles tagged with 5-nm gold (**Fig. 5A** and **B**). These saclike compartments were similar to those seen with VX-LDL. These experiments confirmed that the saclike compartments contained PLC-LDL-gold from the incubating medium bathing the cells. The particle size of the PLC-LDL was much more uniform than with the VX-LDL aggregates. All the particles ranged in diameter between 23 and 37 nm (Fig. 5B).

With a 2-h exposure to PLC-LDL-gold the lipoprotein was also found in numerous small (~357 nm) spherical organelles presumed to be endosomal/lysosomal compartments. These compartments were clearly seen in thin-sectioned conventional fixed tissue (**Fig. 6A**) as well as in the ultrarapidly frozen freeze-etched material (Fig. 6B). The conventionally fixed and thin-sectioned preparations were useful in visualizing these small endosomal compartments because the gold tag within the lumen was always visible. The freeze-fracture images were mostly of the outer surface and not the contents of the endosome. This is because the fracture plane passes over the surface of the small spherical endosome rather than through it.

Some endosomal/lysosomal compartments in macrophages from the PLC-LDL-gold preparations contained small tubular extensions that were devoid of the ingested LDL-gold particles, indicating the possibility of selective ligand sorting within the endosome (**Fig. 7A**). Similar tubular arms of endosomal compartments have been previously described in alveolar macrophages (25) and in hepatic parenchymal cells (26). In freeze-fracture preparations these tubular extensions of the endosomal-sized vesicular structures were commonly seen in both the VX-LDL and PLC-LDL preparations (Fig. 7B) and occasionally in control preparations.

Another striking change in the ultrastructure of the monocyte-macrophages as a result of the incubation with aggregated LDL (either VX-LDL or PLC-LDL) was the increase in the density of tubules within the cytoplasm of the cell. These tubules had diameters of ~86 nm (range, 75–120 nm) and varied from straight cylinders as long as several micrometers to curving tubules of shorter lengths weaving through the cytoplasm. In cells exposed to monomeric native LDL the density of tubules was 1 tubule/μm^2 ($\pm$0.83) (**Fig. 8A**), whereas in the VX-LDL-treated cells (12 h) the cells had 4.86 tubules/μm^2 ($\pm$1.47) (Fig. 8B). In cells exposed to PLC-LDL (2 h) the cells had 3.14 tubules/μm^2 ($\pm$1.21) within the cytoplasm (Fig. 8C).

The freeze-etch micrographs produce images of the tubules from a three-dimensional perspective. The tubules are surrounded by vesicles similar in size to endosomal/lysosomal compartments (160–428 nm). In addition, the tubules were frequently linked to each other and to the vesicles via an intricate network of cytoskeletal filaments. These cytoskeletal filaments were clearly visible in the replicas (**Fig. 9A**). Freeze-etch microscopy of ultrarapidly frozen cells retains the complex cytoarchitecture of structures such as the actin and microtubular filaments of the cytoskeleton. It was also apparent that the tubules within the cytoplasm of the macrophages were frequently in close proximity to clathrin-coated vesicles anchored to cytoskeletal filaments in the cytoplasm (Fig. 9B).

In cells exposed to 12-h incubations with VX-LDL occasionally the fracture plane would crack through a large lysosome-like compartment located in the middle of the cytoplasm, near the nucleus. These lysosome-like compartments were filled with lipid particles larger (78–156 nm) than the aggregated VX-LDL sequestered in the saclike compartments connected to the cell surface (**Fig. 10**). These large lysosome-like compartments were often in close proximity to the cytoplasmic tubules. A tubule in direct contact with the contents of the lysosome is shown in Fig. 10.

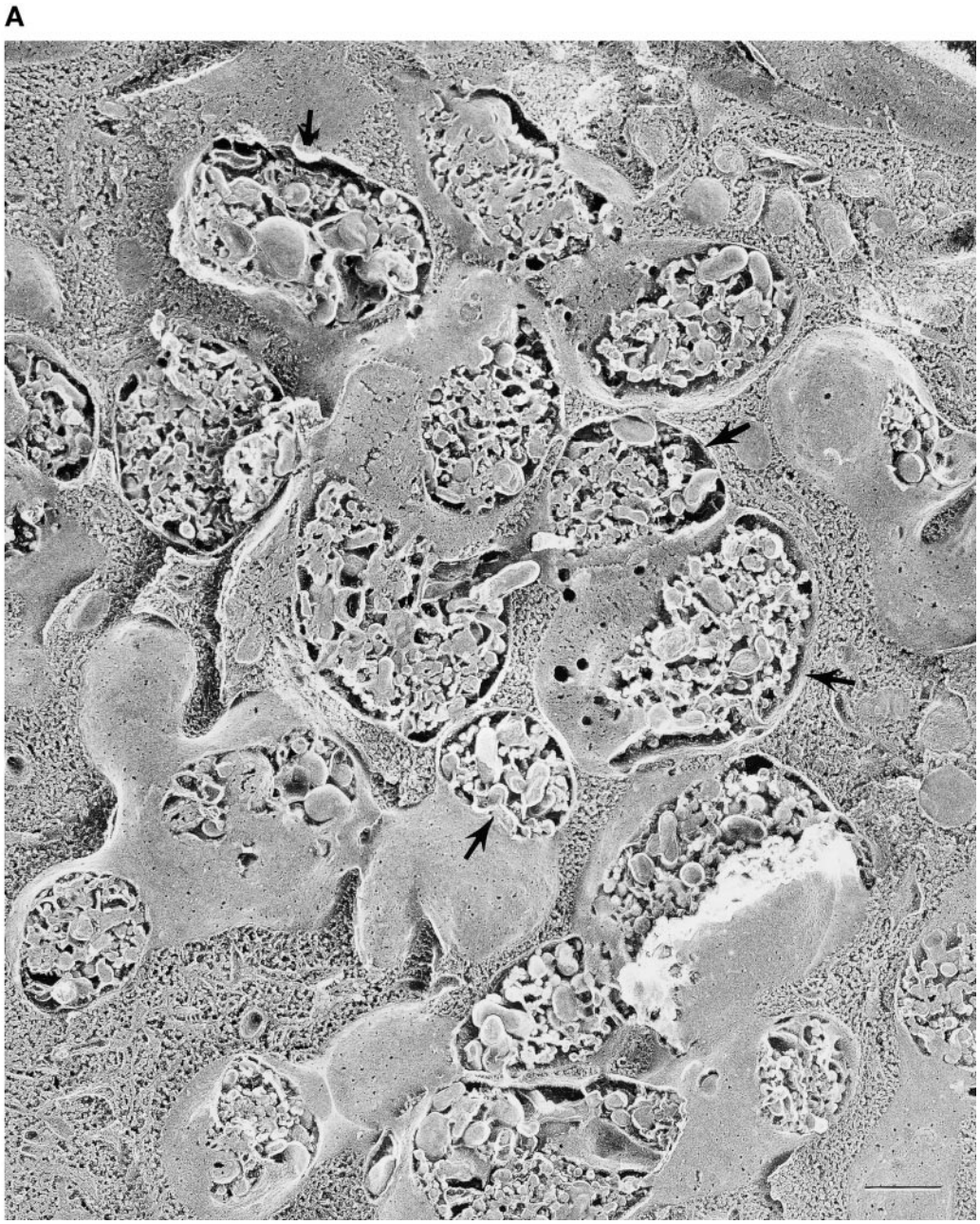
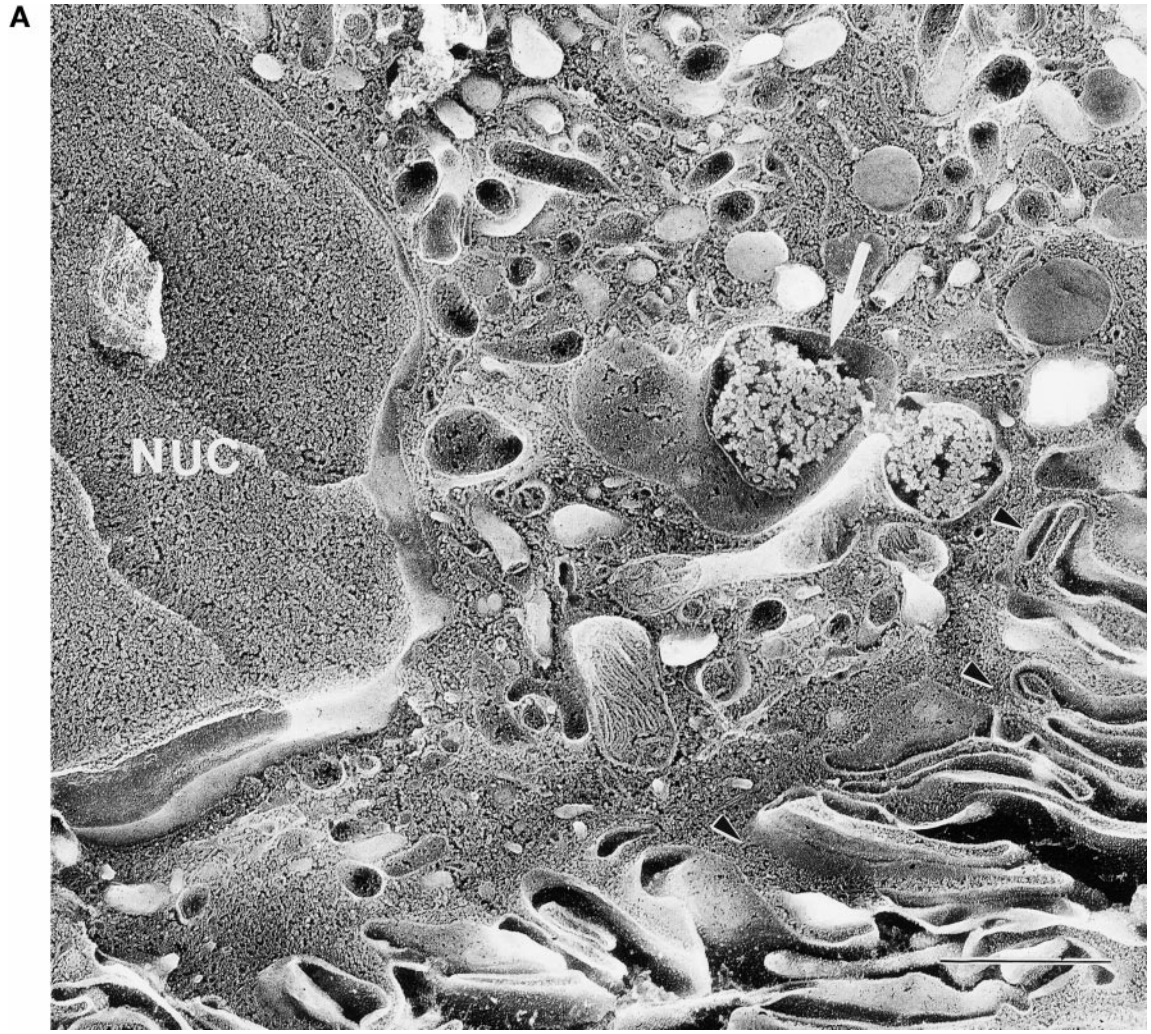


Fig. 4. Freeze-etch electron micrographs of a human monocyte-macrophage incubated for 12 h with VX-LDL. Cells were prepared as described in Fig. 3, but were incubated for 12 h with VX-LDL (500 μg/ml). A: Fracture plane breaks through the cell surface to expose several saclike compartments filled with aggregated lipids (arrows). Original magnification: ×20,136. B: A high magnification freeze-etch electron micrograph of a surface-connected tubule that leads into a saclike compartment within the monocyte-macrophage. The lumen of the sac is filled with aggregated VX-LDL. Arrow is located in the tubule lumen. Original magnification: ×68,344.



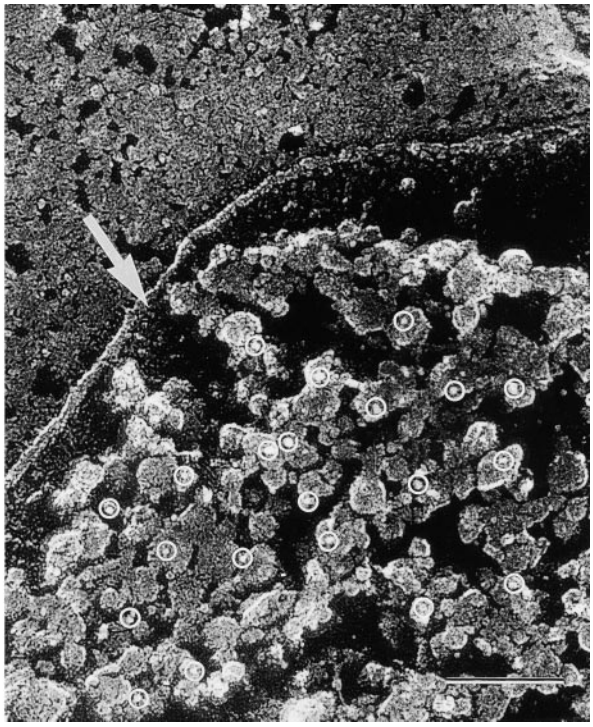


Fig. 5. Freeze-etch electron micrograph of a human monocyte-macrophage incubated for 2 h with gold-tagged LDL enzymatically treated with phospholipase C (PLC)-treated LDL. Cells were maintained for 11 days in culture and incubated for 2 h with gold-tagged PLC-treated LDL (500 μg/ml) as described in Materials and Methods. A: The cell surface is seen at lower right. The border of microvilli is indicated by the arrowheads. The compartment just under the surface of the cell is filled with PLC-treated LDL (arrow). The aggregated clumps of lipid within this cellular compartment are clearly identified by the gold tag on the lipid particles. NUC, Nucleus. Original magnification: ×21,120. B: High magnification of a portion of the intracellular compartment filled with the aggregated PLC-treated LDL-gold presented in (A). The gold tags are circled for easy identification. The arrow points to the cell membrane that defines this compartment. Original magnification: ×148,230.

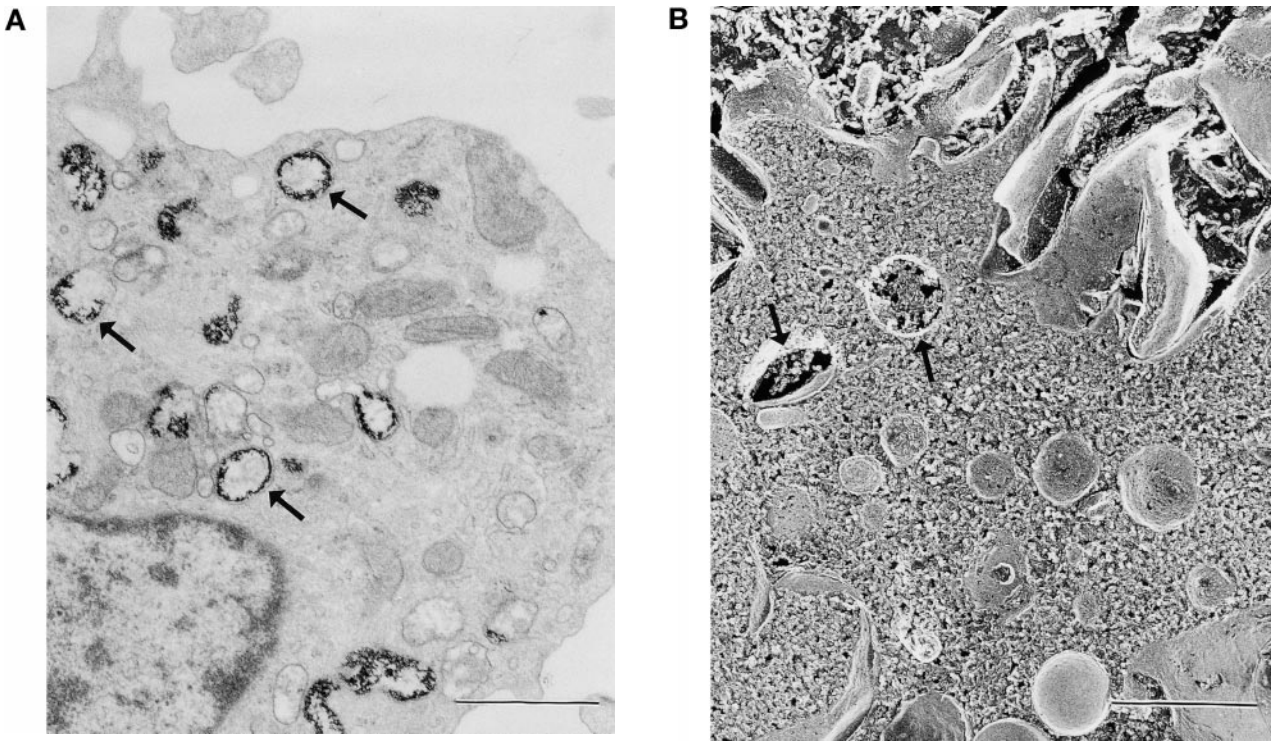


Fig. 6. Two perspectives offered by electron microscopy. Cells were maintained for 11 days in culture and incubated for 2 h with gold-tagged PLC-treated LDL (500 μg/ml). Cell monolayers were prepared for visualization by thin-section electron microscopy (A) or by freeze-etch electron microscopy (B). In (A), the gold-tagged lipoprotein was present in numerous compartments within the cells, including coated and uncoated vesicles and multivesicular bodies, but was most plentiful in endosomal/lysosomal-like compartments indicated by the arrows. Most of these compartments have the gold-tagged lipoprotein distributed around the periphery of the compartment. However, in some compartments the gold-tagged particles fill the entire lumenal space. Original magnification: ×18,400. The freeze-fracture electron micrograph (B) shows a portion of a cultured monocyte-macrophage from the same experiment. The ruffled cell border is seen at the top. Within the cytoplasm are numerous vesicular compartments, two of which have been fractured through their lumens (arrows), exposing their contents of aggregated lipoprotein particles. Original magnification: ×18,000.

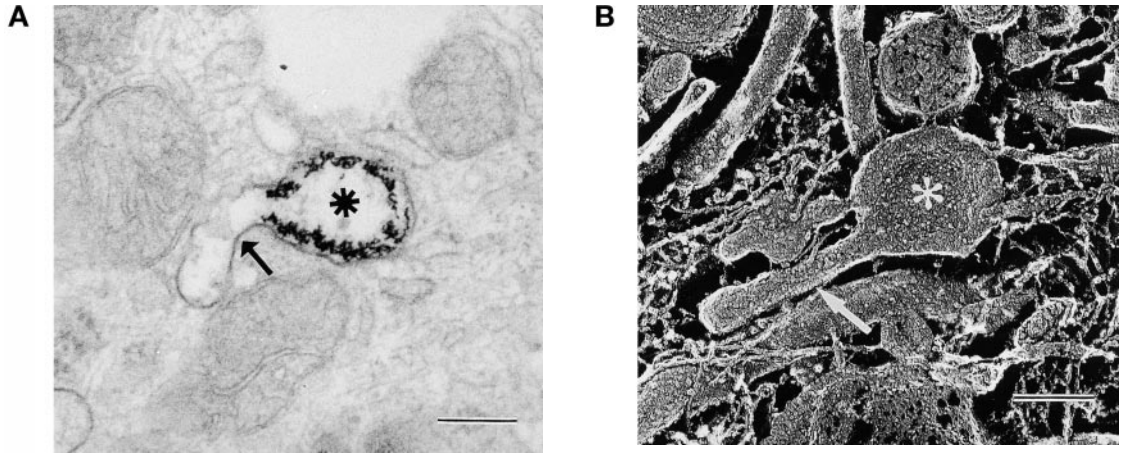


Fig. 7. Endosomal/lysosomal compartments with tubular extensions. A: Human monocyte-macrophages were maintained for 11 days in culture and incubated for 2 h with gold-tagged PLC-treated LDL (500 μg/ml). High magnification thin-section micrograph shows an endosomal/lysosomal compartment with a tubular extension (arrow). Note the tubular extension is free of PLC-treated gold-tagged LDL. The spherical portion of this compartment is lined along its periphery with gold-tagged particles (asterisk). Original magnification: ×45,900. B: Human monocyte-macrophages were maintained for 11 days in culture and incubated for 12 h with VX-LDL (500 μg/ml). The freeze-fracture electron micrograph shows an endosomal/lysosomal compartment with a tubular extension (arrow). As is the case most often with freeze-fracture, the plane of fracture has stayed in the membrane of the compartment and thus the inside of the compartment is not visible. Original magnification: ×45,900.

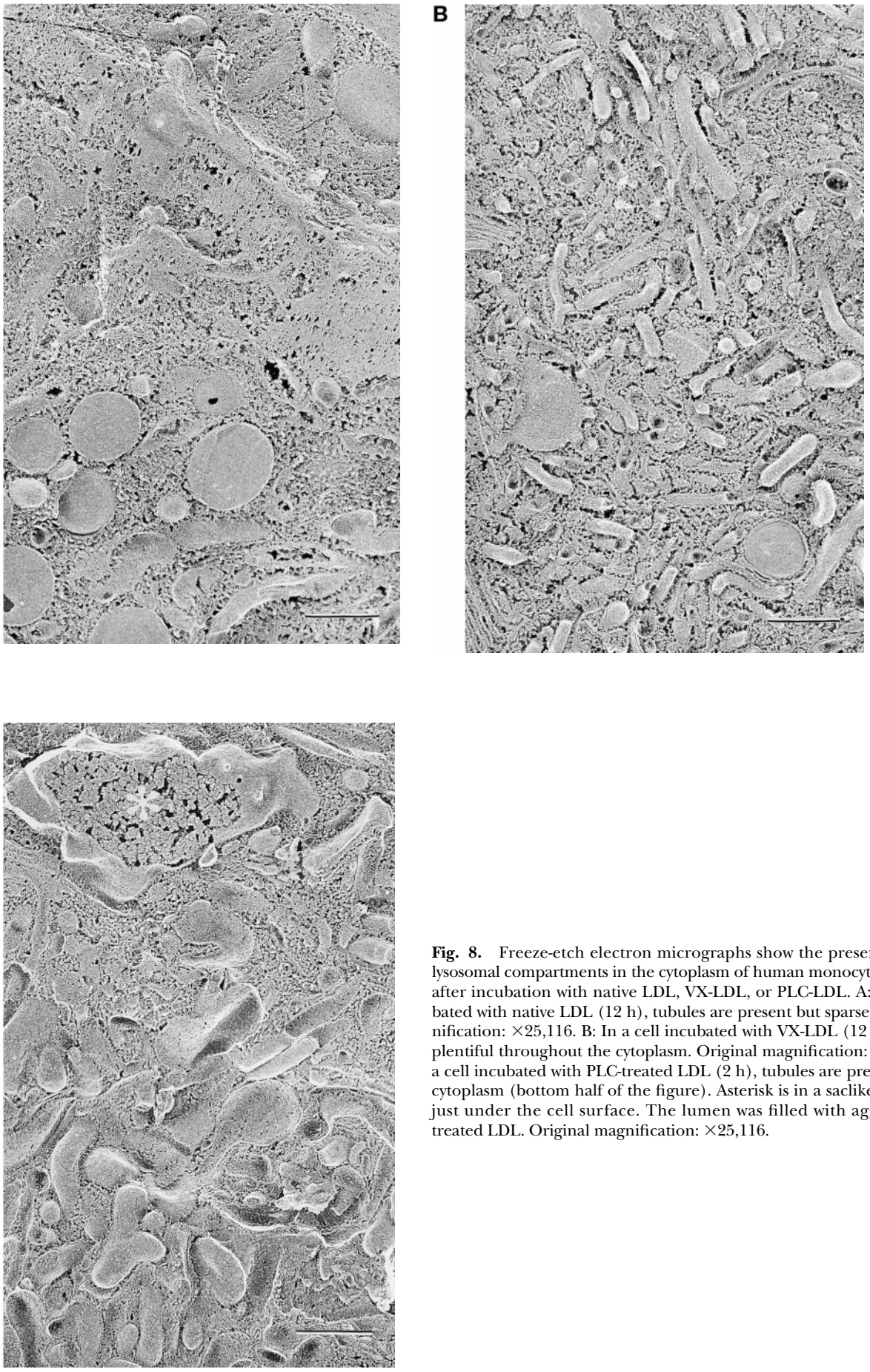


Fig. 8. Freeze-etch electron micrographs show the presence of tubular-lysosomal compartments in the cytoplasm of human monocyte-macrophages after incubation with native LDL, VX-LDL, or PLC-LDL. A: In a cell incubated with native LDL (12 h), tubules are present but sparse. Original magnification: ×25,116. B: In a cell incubated with VX-LDL (12 h), tubules are plentiful throughout the cytoplasm. Original magnification: ×25,245. C: In a cell incubated with PLC-treated LDL (2 h), tubules are present within the cytoplasm (bottom half of the figure). Asterisk is in a saclike compartment just under the cell surface. The lumen was filled with aggregated PLC-treated LDL. Original magnification: ×25,116.

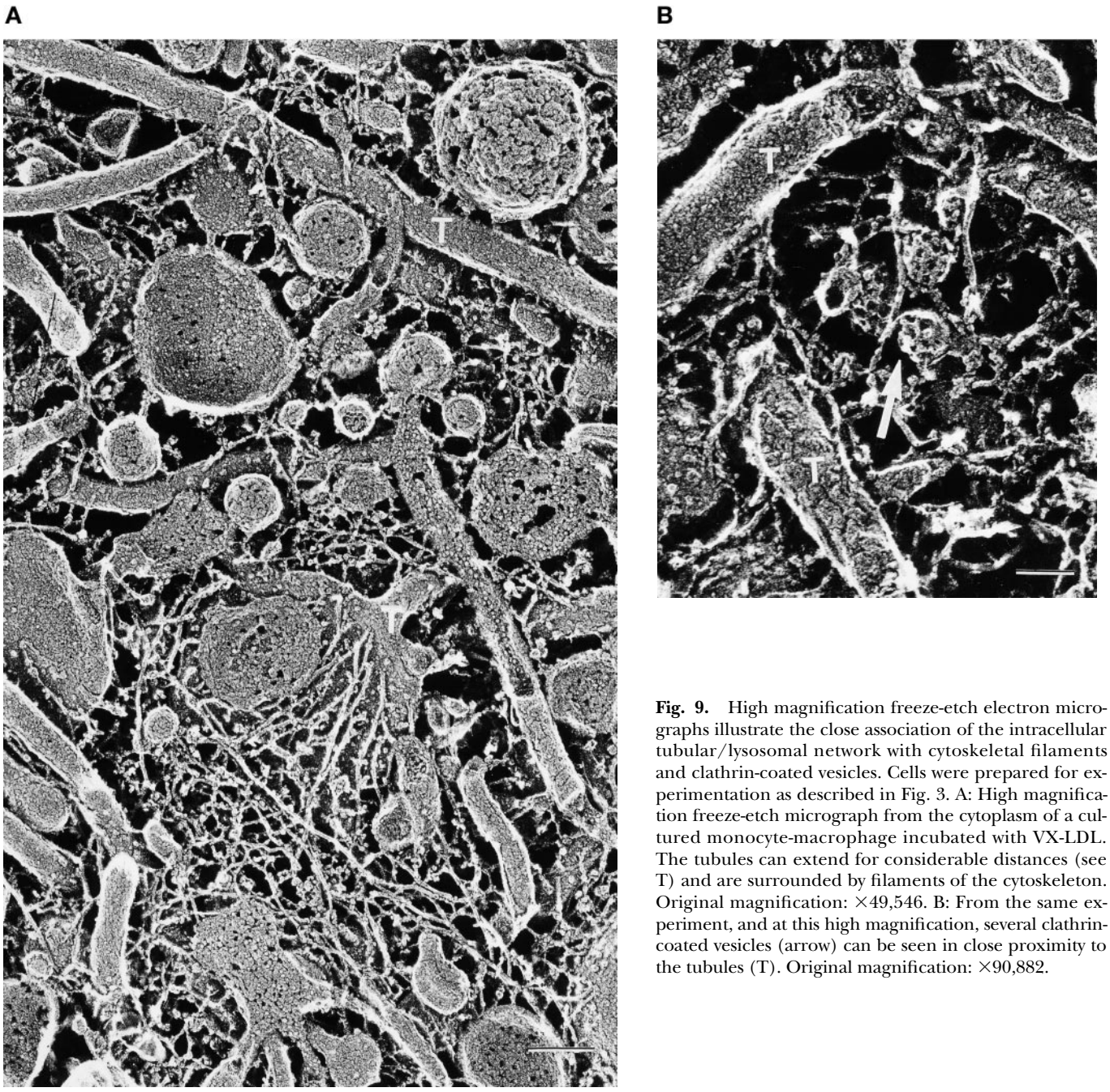


Fig. 9. High magnification freeze-etch electron micrographs illustrate the close association of the intracellular tubular/lysosomal network with cytoskeletal filaments and clathrin-coated vesicles. Cells were prepared for experimentation as described in Fig. 3. A: High magnification freeze-etch micrograph from the cytoplasm of a cultured monocyte-macrophage incubated with VX-LDL. The tubules can extend for considerable distances (see T) and are surrounded by filaments of the cytoskeleton. Original magnification: ×49,546. B: From the same experiment, and at this high magnification, several clathrin-coated vesicles (arrow) can be seen in close proximity to the tubules (T). Original magnification: ×90,882.

DISCUSSION

In macrophages many subcellular compartments have a role in transferring molecules from outside the cell to lysosomes within the cytoplasm (25, 27). These subcellular compartments include coated and uncoated vesicles, early and late endosomes, multivesicular bodies, and tubular/reticular lysosomes, and each compartment appears to be involved in processing lipoproteins within macrophages (28–32). Recently the entry of lipoprotein particles via surface-connected tubules (STEMs) (13, 33) or surface-connected compartments (SCC) (12) has been described. Our understanding of the role of these various endocytic pathways in lipoprotein transport and their relationship to each other in the development of foam cells is still incomplete. The ultrastructural morphology of the various compartments involved in LDL trafficking has been limited to two-dimensional images produced after extensive chemical fixation, staining, and embedding techniques have been applied. The application of freeze-etch technology to generate images of the subcellular compartments in macrophages involved in the uptake of LDL reveals a three-dimensional cell interior filled with complex structures and an elaborate network of filaments. This dynamic view of subcellular structure at the high resolution of the electron microscope provides an additional framework to better understand how lipoprotein particles are transported into, and processed within, macrophages.

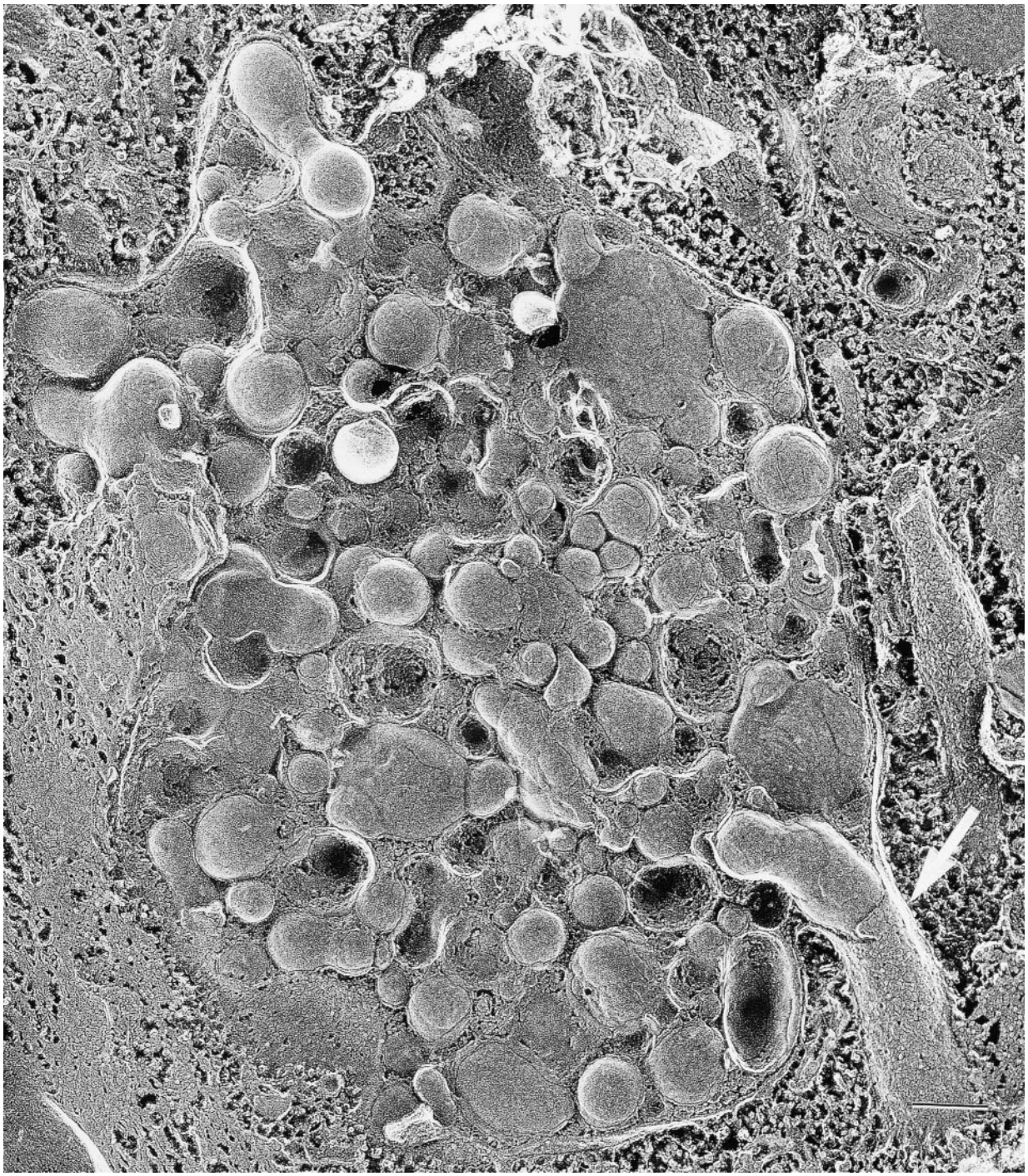


Fig. 10. High magnification freeze-etch electron micrograph of an intracellular compartment in human monocyte-macrophages incubated with VX-LDL. Cells were maintained for 11 days in culture and incubated for 120 h with VX-LDL (500 μg/ml). This high magnification freeze-etch electron micrograph illustrates a lysosomal-like compartment with a tubule in close association in the lower right of the figure (arrow). What is fortuitous is that the fracture plane has cracked through the lumen of both the spherical and tubular portion of this compartment, giving a view of the lumen filled with lipoprotein/lipid particles of various sizes. The tubular lumen also contains lipoprotein/lipid particles. Original magnification: ×57,456.

The present study focused on the ultrastructure of the compartments within the macrophage involved in intracellular trafficking of LDL. The incubation studies with both the isolated cardiac valves and the macrophages grown in culture provide data that LDL uptake occurs quickly. Foam cells could be generated in vitro after 72 h of exposure to aggregated LDL. In isolated cardiac valves after only 4 h of incubation with gold-tagged LDL, the lipoprotein was located in compartments within macrophages including pre-existing lysosomal compartments, saclike structures close to the cell surface, and tubular compartments (Fig. 2). In a previous study we demon-

strated that 2 h after a bolus injection of LDL into the circulation of the rabbit, lipid aggregates could be detected in the intima of the aorta (20). We were also able to show individual LDL particles in the subendothelial matrix, and LDL aligned along collagen fibers in the intima, suggesting that lipid aggregates formed from these particles enmeshed in the matrix (17). These ultrastructural data support the concept that the retention and aggregation of LDL are important early steps for LDL uptake into macrophages and that LDL aggregates can form quickly in vivo within the matrix of the intima.

The series of experiments that exposed cultured human macrophages to aggregated LDL (either VX-LDL or PLC-LDL) were designed to monitor the distribution of the aggregated LDL into endocytic compartments within the cell. One of the most visible compartments was the labyrinth of large saclike structures just under the cell membrane. Because of the size of these sacs and their location under the cell surface, the fracture plane cracked into the lumen, exposing the lipoprotein-filled contents (Figs. 4 and 5) and connection of the lumen to the cell surface (Fig. 4B). These saclike structures, seen here in quasi-three-dimensional detail, are similar to the thin-section images of surface-connected compartments described by Kruth et al. (34, 35) as part of a unique endocytic pathway. What is still unclear is whether the surface-connected compartments serve a storage function, whether lipoprotein within these compartments undergoes metabolic processing, and whether these structures are related to other endosomal/lysosomal compartments.

In addition to the saclike compartments described above, aggregated LDL was present in compartments that resembled the well-described endosomal/lysosomal structures typically found in macrophages (25, 27). The early endosomes and prelysosomal compartments are more spherical and have diameters smaller than the saclike surface-connected compartments. In freeze-etch images the fracture plane rarely breaks through the lumen but rather travels through the membrane of the sphere. In Fig. 6B the fracture broke through the lumen in only two of the endosomal-like compartments. Thin sections of cells that had been incubated with aggregated PLC-LDL tagged with gold provided complementary images. Sections exposed the gold-tagged contents. Most compartments contained gold around the inner periphery of the lumen, whereas in a few compartments the gold was dispersed throughout the lumen (Fig. 6A). A particularly interesting variant of the spherical endosome is the spherical endosomal compartment with a tubular extension. These structures have been described in macrophages (25) and in detail in hepatic parenchymal cells, where the compartment has been called CURL, for compartment of uncoupling of receptor and ligand (26). CURL vesicles appear to accumulate ligand within the lumen, whereas the tubular extensions are free of ligand but do contain receptor. Thus one function of the tubular portions of CURL may be to provide escape of receptor from lysosomal degradation. Spherical endosomal compartments with tubular extensions were present in the cytoplasm in all of the macrophages incubated with LDL. Their distinctive structure is clearly seen in three dimensions in freeze-etch micrographs (Fig. 7B). In thin-section electron micrographs, the ligand, in this case PLC-LDL tagged with gold, is present only in the lumen of the spherical portion of the compartment and is absent from the tubular lumen (Fig. 7A). It is difficult to determine the function of endocytic transport compartments on the basis of morphology alone. However, the presence of these CURL-like compartments in macrophages actively engaged in lipoprotein uptake is an interesting observation.

Lysosomes are usually described as spherical intracellular organelles. Several studies, however, have described a network of tubular lysosomes in cultured human macrophages, mouse peritoneal macrophages, and J774.2 macrophages (36–38). These tubular structures have been identified as lysosomes by acid phosphatase histochemistry and immunofluorescence localization of cathepsin L. The tubules can vary in length but, in some cases, they extend for several microns through the cytoplasm. Tubular lysosomes can be markedly increased by treatment with phorbol 12-myristate 13-acetate (37) and with high cellular rates of accumulation of Lucifer yellow (38).

Interestingly, the maintenance of the tubular lysosome morphology is dependent on an intact cytoskeleton. As Fig. 8 illustrates, cultured human monocyte-macrophages incubated with VX-LDL or PLC-LDL have abundant tubular lysosome-like structures within the cytoplasm. These tubules were especially prominent in cells incubated for 12 h with VX-LDL. Comparison of Fig. 8A with B shows the marked increase in the tubular lysosome structures after incubation with VX-LDL. With the freeze-etch morphology the tubules could be followed for some distance in the cytoplasm. The direct association of filaments of the cytoskeleton with the tubular lysosomes was evident in Fig. 9. Previous studies have indicated that intact microtubules are necessary for tubular lysosome movement and shape. A light microscopic study that preserved the labile tubular lysosome compartment and also allowed immunocytochemical localization of microtubules was able to demonstrate the close spatial relationship between the tubular lysosomes and cytoplasmic microtubules in macrophages (39). The freeze-etch images shown in Fig. 9 reveal a complex picture of lysosome-like tubules enmeshed in a net of microfilaments that are also linked to clathrin-coated vesicles.

The cytoskeletal supramolecular structure has always presented a challenge to morphologists. At the relatively low resolution of optical microscopy, the cytoskeletal networks are often dramatically visible, particularly in confocal images. The high resolution images possible with electron microscopy, however, require extensive cellular extraction techniques. Moreover, most electron microscopic images do not provide the necessary three-dimensional perspective to image any connections the cytoskeletal filaments may have with subcellular structures. The freeze-etch morphology of the macrophage indicates that filaments of the cytoskeletal network are linked to several

subcellular organelles, including the compartments involved in lipoprotein processing.

In the formation of foam cells the mature "end-stage" lysosomes become filled with lipid. These large spherical lysosome compartments can be found deep in the cytoplasm. In freeze-etch images the fracture plane runs through the membrane of the compartment. The lipid contents of the compartment are evident as bulges that deform the smooth outline of the membrane and, when the fracture plane cracks into the lumen of the compartment, the surface of the aggregated lipid particles is seen (Figs. 1 and 10). The structural features of the lipid contents in these compartments were first clearly demonstrated with freeze-etch replicas at the resolution of the electron microscope in a study of advanced lesions in Watanabe heritable hyperlipidemic rabbits by Amanuma et al. (40). What is still unclear is the relationship between the end-stage lysosomes and the extensive tubular lysosomes that are present when the macrophages are exposed to VX-LDL or PLC-LDL. Do the tubular lysosomes have direct contact with the end-stage lysosome? Unfortunately, freeze-etch morphology cannot definitely answer this question. Figure 10 does pose an interesting possibility that there is a direct connection between these compartments, and that the tubular lysosomes may deliver lipid particles to the final lysosome compartment.

The ultrastructural data presented here support the concept that sequestration of aggregated LDL by macrophages involves many pathways. The three-dimensional ultrastructure of a complex network of coated and uncoated vesicles, surface-connected saclike compartments, endosomal/lysosomal vesicles, and tubular lysosomes all enmeshed in the microtubular, microfilament cytoskeletal network has been presented by the use of ultrarapid freezing and freeze-etch techniques. Many questions about the interactions and functions of these compartments remain. However, this study affords a new and deeper insight into macrophage cell structure during the critical stage of sequestration of aggregated LDL and the development of a foam cell.

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