Virchows Arch A (1986) 410:231–238

Virchows Archiv A

Studies on fine structure and location of lipids in quick-freeze replicas of atherosclerotic aorta of WHHL rabbits

Kimiko Amanuma [1]*, Toku Kanaseki [2], Yohko Ikeuchi [2], Shoji Ohkuma [1], and Tatsuya Takano [1]

[1] Department of Microbiology and Molecular Pathology, Faculty of Pharmaceutical Sciences, Teikyo University, Sagamiko, Kanagawa 199-01, and

[2] Department of Genetics, Tokyo Metropolitan Institute for Neurosciences, Fuchu, Tokyo 183, Japan

Summary. The fine structure of intracellular and extracellular lipids in the atherosclerotic aorta of Watanabe-heritable hyperlipidemic (WHHL) rabbits was demonstrated by a quick-freeze etching technique. Many lipid droplets, with and without a membrane, were observed in the foam cells. Membrane-free droplets were observed as onion-like structure with a concentric lamellar structure surrounded by 10 nm filaments. Droplets surrounded by a limited membrane probably correspond to lipid-laden lysosomes.

In the extracellular connective tissue space, marked accumulation of lipids with a vesicular structure was seen among collagen fibers. The appearance of these lipids was similar to that of lipids in lysosomes of foam cells.

Key words: Atherosclerosis – Quick-freeze replica – Lipid deposit – Foam cell – Extracellular lipid

Introduction

A prominent feature of atherosclerosis is marked accumulation of lipids, especially cholesterol esters, in the arterial wall (Portman 1970). These lipids are observed as small droplets with intense anisotropy by polarized microscopy (Ghidoni and O'Neal 1976). They have been isolated (Lang and Insull 1970; Takano et al., 1982), and their biochemical (Lang and Insull 1970; Takano et al. 1982) and morphological characteristics (Hata et al. 1974; Insull et al. 1974; Lang and Insull 1970) have been examined. They consist mainly of cholesterol ester (about 95%) and have been called cholesterol ester-rich lipid inclusions (Hata et al. 1974). Models for organization of cholesterol ester were proposed from their optical characteristics (Hata et al. 1974) and their X-ray diffraction pattern (Engelman and Hillman 1976).

Histochemical studies, for example using oil-red O staining (Geer and Haust 1972), have demonstrated that in early lesions lipids accumulate mainly in the cytoplasm of cells, but in more advanced lesions they are also found in the extracellular matrix. Cytochemical studies by the thin section method of electron microscopy have shown that the intracellular lipids are stored in two forms in the cytoplasm of foam cells: membrane-bound vacuoles, which probably correspond to lysosomes, and vacuoles without a peripheral membrane (Coltoff-Schiller et al. 1976; Goldfischer et al. 1975; Shio et al. 1974; Shio et al. 1979). However, by thin section electron microscopy, lipid droplets are recognized only as vacuoles in which the lipids had been present since neutral lipids are extracted in the dehydration process. For this reason, the structural characteristics of lipids in foam cells have not yet been well elucidated.

In this work, we examined the fine structure of advanced lesions of fatty streak in the atherosclerotic aorta of WHHL rabbits (Watanabe 1980) by a quick-freeze, etching technique (Heuser 1981) to preserve lipid structures in foam cells and in extracellular spaces.

Materials and methods

Male homozygous WHHL rabbits about 12 months old were anesthesized, and advanced lesions of the white fatty streak type were removed from thoracic aortas. The tissues were immediately quick-frozen by slamming them against a liquid-helium cooled copper block according to Heuser's cryotechnique (Heuser 1981). Frozen samples were fractured by a Balsers' BAF 300 freeze-fracture machine. Fractured samples were deep-etched by warming them to −95° C at a vacuum of

Offprint requests to: T. Takano at the above address

* *Present address:* Cell Sci. Tech. Div., Ferm. Res. Inst., Tsukuba, Ibaraki 305, Japan

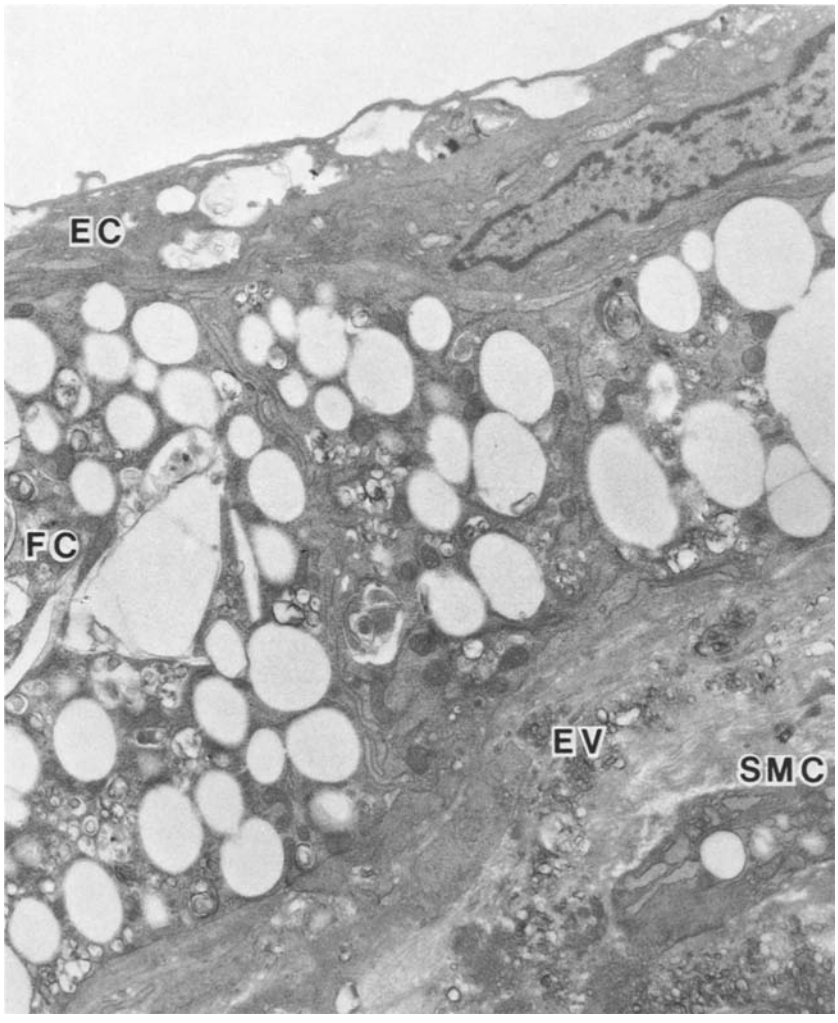


Fig. 1. Low magnification view (× 5,900) of the atherosclerotic aorta of a WHHL rabbit. Typical foam cells (*FC*) and a smooth muscle cell (*SMC*) containing vacuoles are seen in the subendothelial area. Vesicles (*EV*) are also seen in the extracellular space filled with collagen fibers. (*EC*), endothelial cell

2×10^{-6} torr for 15 min. They were rotary replicated with platinum-carbon at an angle of 25°. Tissues less than 20 nm depth from the endothelial surface was examined. For thin section electron microscopy, similar portion of the lesions from WHHL rabbits, were fixed with Karnovsky's fixative (Karnovsky 1965) for 1 h followed by phosphate buffered 1% OsO_4. Replicas and thin sections were examined at 200 KV in JEOL 200 B and JEOL 200 CX electron microscopes.

Results

A low magnification view of an advanced lesion of fatty streaks of WHHL rabbits is shown in Fig. 1. These lesions were observed by the quick-freeze, etching method. The cytoplasm of foam cells was found to be filled with onion-like granules (0.7–1.7 μm in diameter) with a lamellar structure (Fig. 2). A small convex or concave part (about 0.05 μm) is seen in the center of cross-fractured granules, the profiles of which looks like cymbals. No limiting membrane is seen around each onion-like granule. In the cytoplasm, smaller granules (0.1–0.6 μm) (arrowheads) which must be lysosomes were surrounded by a membrane. Mitochondria and elements of endoplasmic reticulum were rarely seen in these foam cells.

Examination of these foam cells by the thin section method clearly shows that electron-lucent vacuoles (Fig. 3) corresponded to the granules with onion-like lamellae seen in quick-freeze replicas. These findings indicate that the components of onion-like granules are alcohol extractable materials, such as lipids. Many 10 nm filaments are seen at the boundaries of electron-lucent vacuoles (Fig. 3, arrowheads). Filamentous structure is also seen around these lipid droplets by the quick-freeze etching technique (Fig. 3, inset). Vacuoles with a limiting membrane in section (Fig. 3) correspond to the smaller granules surrounded by a membrane in replicas (Fig. 2, arrowheads). These observations indicate that the organelles, probably lipid-laden lysosomes, contain small granules.

Figure 4 shows an grape-like organelle contain-

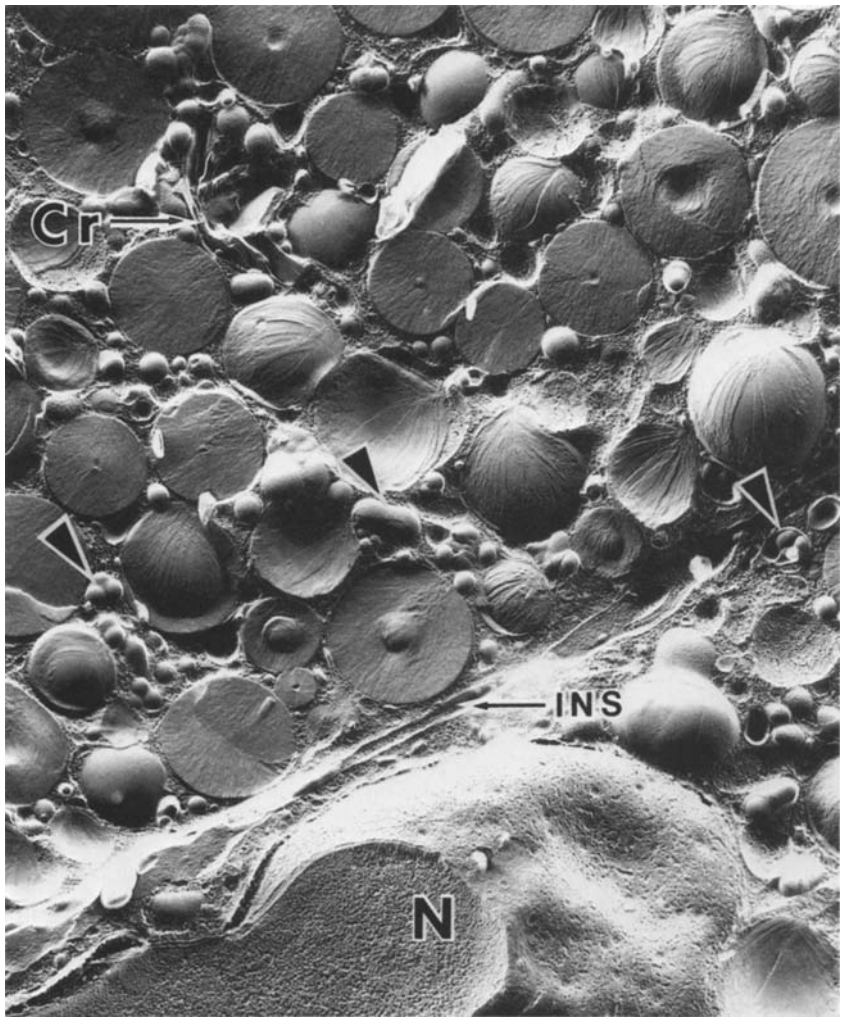


Fig. 2. Quick-freeze replica of two adjacent typical foam cells. The cytoplasm is filled with numerous granules with an onion-like lamellar structure. Smaller granules surrounded by a membrane (*arrowheads*) are seen among these onion-like granules. A few plate crystals (*Cr*) are also seen. (*N*), nucleus; (*INS*), intercellular space. (×13,700)

ing various sized granules in a foam cell. The organelle is surrounded by a single membrane, and part of the membrane shows an E-fracture face, on which many membrane particles are seen. The grape-like organelle contained a number of lipid droplets surrounded by a membrane, suggesting that this organelle is a lysosome containing lipid droplets which corresponds to a "multivesicular body" by thin section electron microscopy.

In the cell containing a few lipid droplets, the various kinds of organelles, such as lysosomes, mitochondria, endoplasmic reticulum, ribosomes and cytoplasmic filaments are observed (Fig. 5).

Smooth muscle cells containing large electron-lucent vacuoles were also seen in the subendothelial layer of the lesions (Fig. 6). The correspondence of these electron-lucent vacuoles with the onion-like droplets is clearly seen by comparison with quick-freeze replicas (Fig. 6, inset). Although the cell shown in Fig. 6 seems entirely different from those shown in Fig. 2, these cells contain the similar onion-shaped lipid droplets.

In the extracellular connective tissue space, a large number of irregular vesicles (Fig. 7, arrow) are seen among what appear to be collagen fibers from their characteristic repeating pattern. The surface appearance of these vesicles looks like freeze-fractured biomembrane faces, and concave fracture faces were observed, suggesting that some vesicles are covered by a membrane. These vesicular structures clearly correspond to vesicles rimmed with an unit membrane structure (Fig. 7, inset, arrow) and electron-lucent regions among collagen fibers in thin sections. These observations suggest that these vesicles consist mainly of lipids, and that some of them are covered by a membrane.

On thin section electron microscopy, the cytoplasm of some lipid-filled foam cells was seen abnormally concentrated and their cytoplasmic membrane was broken. These cells often contained

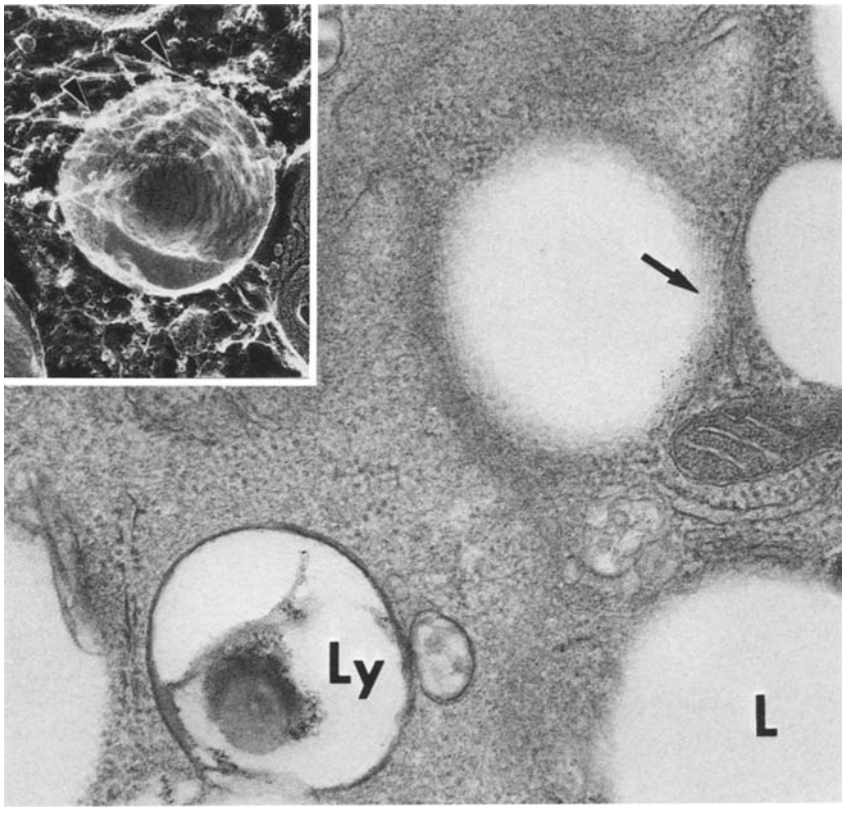


Fig. 3. Thin section of the cytoplasm of a foam cell. Lipid droplets are observed as vacuoles with (*Ly*) and without (*L*) membranes. Some membrane-free droplets are observed surrounded by 10 nm filaments (*arrows*). These filaments (*arrowheads*) are also seen associated with onion-like droplets in quick-freeze replicas (*inset*). (×29,000; inset, ×47,000)

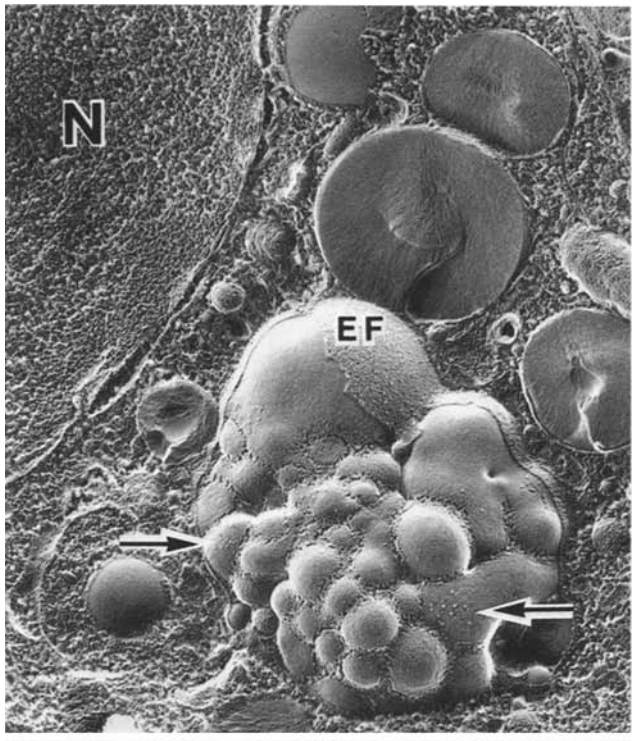
large crystal plates like that in the quick-freeze replica shown in Fig. 8. The extracellular space shown in Fig. 9 contains plate crystals and numerous irregular vesicles. Some vesicles are clustered and aggregated together (Fig. 9), and clustered vesicles are similar in size to the cells and seem to be covered by a membrane.

Discussion

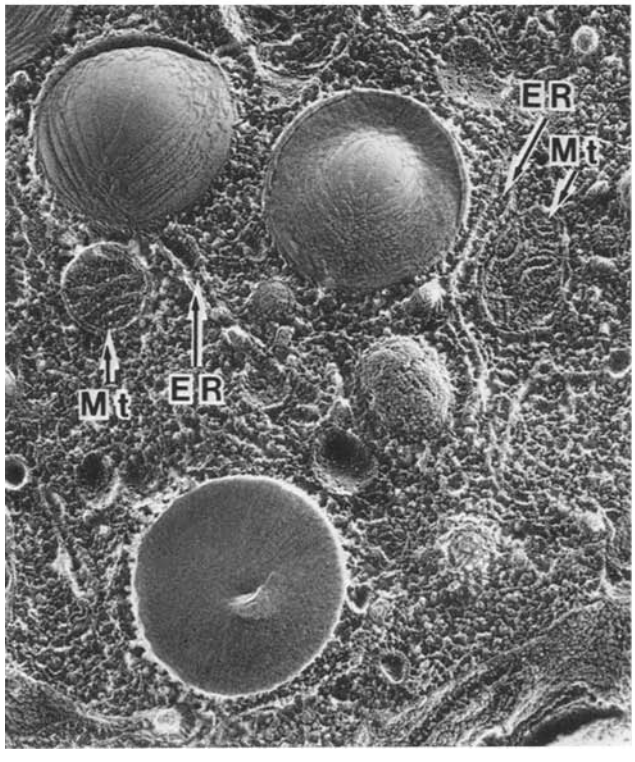
In this work we demonstrated the structure of lipid droplets in foam cells by a quick-freeze etching technique. We also demonstrated, for the first time, ultrastructural characteristics of extracellular vesicles containing lipids, which have previously been shown to exist by lipid staining of sections and their examination by light microscopy (Geer and Haust 1972).

Several groups have observed by thin section electron microscopy that lipid droplets in foam cells are present both as membrane-bound vacuoles, which may correspond to lysosomes, and membrane-free droplets (Coltoff-Schiller et al. 1976; Goldfischer et al. 1975; Shio et al. 1974; Shio et al. 1979). In the present work, these lipid droplets with and without membranes were also observed in quick-freeze replicas of foam cells which may be derived from macrophages (Figs. 2, 4) and smooth muscle cells (Fig. 6). Membrane-free droplets were more numerous than membrane-bound droplets. The membrane-free droplets appeared to consist of onion-like concentric lamellae. Similar onion-like lamellae were previously observed by the conventional freeze-fracture method (Ruska et al. 1972). These findings suggest that the constituent lipids of the droplets are in an orderly arrangement as lamellae. This molecular arrangement of lipids is consistent with the model proposed by Hata and Insull (1973) and Engelman and Hillman (1976) from the anisotropic nature and X-ray diffraction pattern of cholesterol ester-rich inclusions. Onion-like lamellae were observed in anisotropic cholesterol ester droplets prepared in vitro (Lundberg 1975). These results suggest that onion-like droplets consist mainly of cholesterol ester and correspond to the cholesterol ester-rich lipid inclusions isolated by Lang and Insull (1970) and by us (Takano et al. 1982).

More detailed examination of the membrane-free droplets by the thin section and the quick-freeze etching method showed that these onion-like lipid droplets were surrounded by 10 nm filaments (Fig. 3). Existence of similar filaments was observed in lipid droplets of cholesterol-loaded mac-



4



5

Fig. 4. Quick-freeze replica of the perinuclear area of a foam cell. An organelle containing various sized granules is seen among onion-like droplets. Part of the membrane of this organella shows an E-fracture face (*EF*). The fractured face of some granular contents are smooth and that of others contains membrane particles (*arrows*). (*N*), nucleus (× 20,000)

rophages (McGookey and Anderson 1983) and in triglyceride droplets in adipocytes (Luckenbill and Cohen 1966; Wood 1967).

The other type of lipid droplets in foam cells is membrane-bound (Figs. 2, 4) observed in lysosomes. These lipid-laden organelles may correspond to low-density lysosomes, papered by flotation sucrose density gradient centrifugation (Peters et al. 1973; Takano et al. 1981). Lipid droplets in lysosomes vary in size.

In early stage of foam cell transformation, membrane-free droplets may accumulate by re-esterification of free cholesterol in the endoplasmic reticulum (ER) after their hydrolysis in lysosomes as shown to be the case in cholesterol-loaded macrophages (Brown et al. 1979). However, elements of the ER are rarely found in highly lipid-filled foam cells (Fig. 2). Another possible explanation for the accumulation of membrane-free droplets in these highly lipid-filled foam cells is that these droplets are produced from lysosomes or multivesicular bodies (Fig. 4) (Smith and Farquhar 1966; Wake 1974). This possibility must be examined by investigating the transition from membrane-bound to membrane-free droplets.

In the extracellular space of the atherosclerotic aorta, the deposition of lipids has been shown by light microscopic studies (Geer and Haust 1972). By thin section electron microscopy, several investigators have suggested that the electron dense particles correspond to extracellular lipids (Geer and Haust 1972; Marshall et al. 1966). However, the fine structure of extracellular lipids has not been shown due to the limitation of the thin section method; that is that neutral lipids are extracted during dehydration procedure. By a quick-freeze and etching technique, we clearly demonstrated the ultrastructural characteristics of extracellular lipids; they appeared in the form of irregular-shaped vesicles and some of them were observed to have a membrane on their surface (Figs. 7, 9). The vesicles and electron-lucent parts among collagen fibers in thin sections (Fig. 7, inset) corresponded to these extracellular lipids. Some extracellular vesicles were clustered and the clustered vesicles were similar in size to the cells (Fig. 9). In the lipid-filled cells which had been ruptured or were destined to collapse in the atherosclerotic aorta (Fig. 8), some of extracellular vesicles containing lipids were derived from disrupted foam cells. At the

Fig. 5. Cytoplasm of a foam cell containing a few lipid droplets by the quick-freeze etching technique. Mitochondria (*Mt*) and endoplasmic reticulum (*ER*) are seen. (× 40,000)

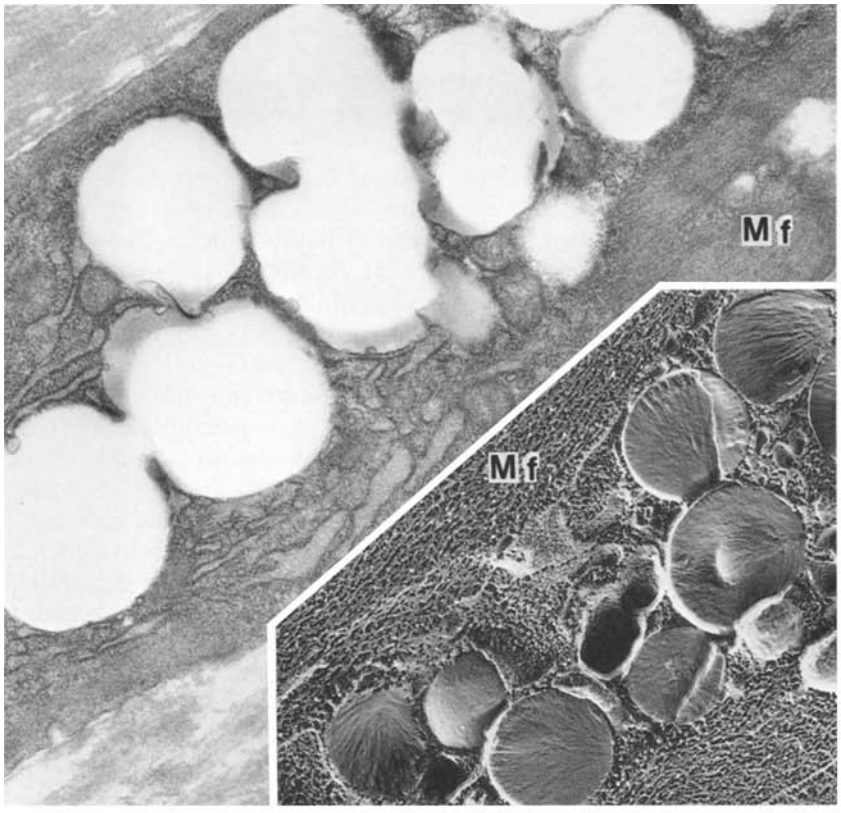


Fig. 6. Thin section of a lipid-laden smooth muscle cell in the subendothelial space of atherosclerotic aorta. Myofilaments (*Mf*), endoplasmic reticulum, and large electron lucent vacuoles are seen in the cytoplasm by the thin section method. These vacuoles correspond to the onion-like droplets in smooth muscle cells in quick-freeze replicas (*inset*). (× 15,000; inset, × 18,000)

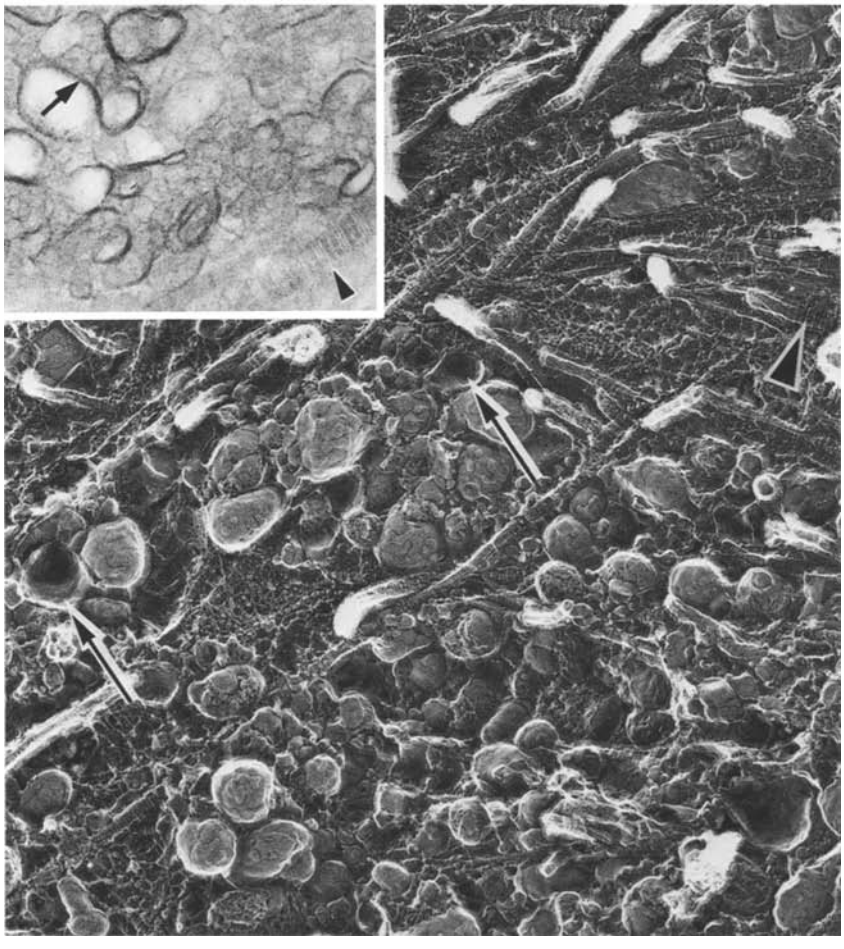


Fig. 7. Appearance of the extracellular connective tissue space of atherosclerotic aorta by the quick-freeze, etching technique. Large amounts of lipids with a vesicular structure are observed among collagen fibers, which show a characteristic repeating pattern (*arrowheads*). Some of these vesicular structures appear to be covered by a membrane. Arrows indicate fractured faces of vesiculated lipids corresponding to the P-fracture face of biomembrane; see text. These vesicular structures correspond to vesicles rimmed with an unit membrane structure (*inset, arrow*) among collagen fibers (*inset, arrowhead*) in thin sections. (× 44,000; inset, × 40,000)

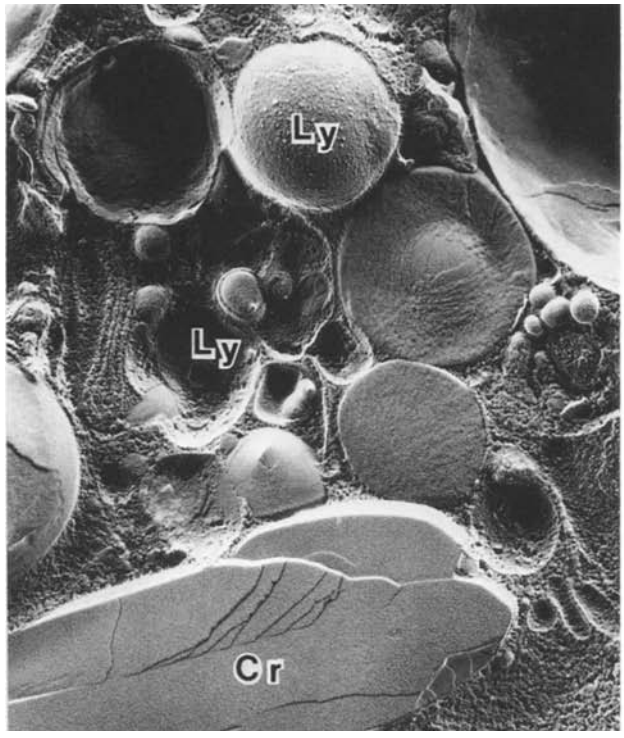


Fig. 8. Appearance of the cytoplasm of a foam cell, which may be destined to collapse, by the quick-freeze etching technique. Onion-like droplets and a plate crystal (*Cr*) are seen. Several lysosomes are observed (*Ly*); some were fractured between their membranes, and some were fractured inside revealing lipid droplets. (× 32,000)

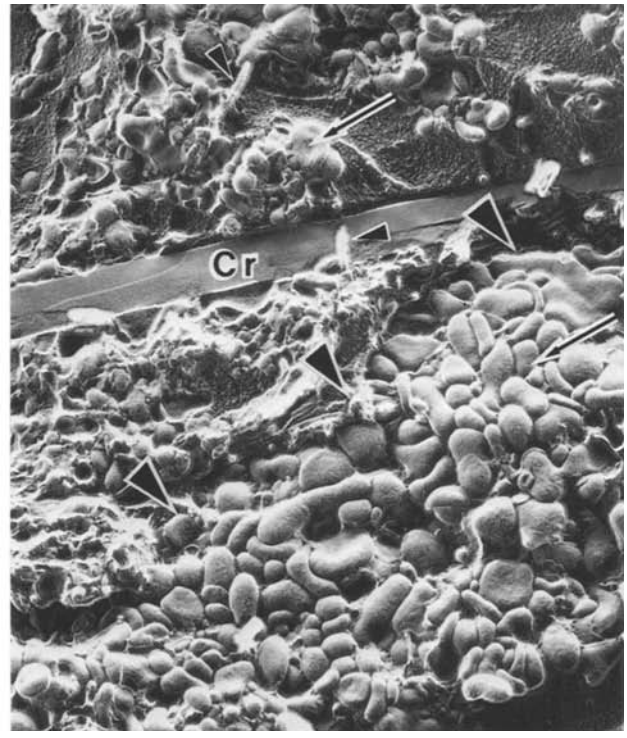


Fig. 9. Appearance of extracellular space of atherosclerotic aorta by the quick-freeze etching technique. A plate crystal (*Cr*) and many vesicular structures are observed. Some of these vesicular structures seem to be covered by a membrane (*arrows*). In the lower right, an aggregation of vesicles is observed (*large arrowheads*). The surface of the aggregate is smooth. Arrowheads indicate collagen fibers. (× 26,000)

same time, lipoprotein or denatured lipoprotein might be also deposited in the extracellular space (Geer and Haust 1972).

These extracellular lipids seem to be endocytized and to accumulate in lysosomes, since the appearance of extracellular lipids (Fig. 8) was very similar to that of lipids in multivesicular body of foam cells (Fig. 4). Brown and Goldstein proposed a scavenger system (1983), and they observed marked accumulation of cholesterol ester in cultured macrophages using modified low-density lipoprotein (Brown et al. 1979). Endocytic activity of foam cells was demonstrated by phase contrast cinemicrophotography (Takano et al. 1984). The extracellular lipids observed as vesicles in this work may correspond to these denatured lipoproteins endocytized by scavenger cells such as macrophages, modified smooth muscle cells and foam cells, and foam cell transformation may be accelerated.

This is the first report of ultrastructural characterization of lipids accumulated intracellularly and extracellularly in atherosclerotic aorta by a quick-freeze etching technique, compared with thin section electron microscopy. Investigations on the role and relationship of intracellular and extracellular lipids are important for elucidating the mechanism of foam cell transformation in atherosclerosis.

Acknowledgements. We thank Dr. Yoshio Watanabe and Dr. Kazushi Hirohata (Kobe University) for providing us with WHHL rabbits and for advice on maintaining these rabbits. We thank Dr. Yoji Yoshida (Yamanashi Medical University) for helpful comments and discussion.

This work was supported by Grants for Scientific Research from the Department of Education, Science and Culture of Japan.

References

Brown MS, Goldstein JL, Krieger M, Ho YK, Anderson RGW (1979) Reversible accumulation of cholesterol esters in macrophages incubated with acetylated lipoproteins. J Cell Biol 82:597–613

Brown MS, Goldstein JL (1983) Lipoprotein metabolism in the macrophage: Implications for cholesterol deposition in atherosclerosis. Ann Rev Biochem 52:223–261

Coltoff-Schiller B, Goldfischer S, Wolinsky H, Factor SM (1976) Lipid accumulation in human aortic smooth muscle cell lysosomes. Am J Pathol 83:39–44

Engelman DM, Hillman GM (1976) Molecular organization of the cholesteryl ester droplets in the fatty streaks of human aorta. J Clin Invest 58:997–1007

Geer JC, Haust MD (1972) Smooth muscle cells in atherosclerosis. Monographs on atherosclerosis vol. 2, S Karger, New York

Ghidoni JJ, O'Neal RM (1976) Recent advances in molecular pathology. A review: ultrastructure of human atheroma. Exp Mol Pathol 7:378–400

Goldfischer S, Schiller B, Wolinsky H (1975) Lipid accumulation in smooth muscle cell lysosomes in primate atherosclerosis. Am J Pathol 78:497–504

Hata Y, Insull W, Jr (1973) Significance of cholesterol esters as liquid crystal in human atherosclerosis. Jpn Circ J 37:269–275

Hata Y, Hower JH, Insull W, Jr (1974) Cholesterol ester-rich inclusions from human aortic fatty and fibrous plaque lesions of atherosclerosis. Am J Pathol 75:423–456

Heuser J (1981) Preparing biological samples for stereomicroscopy by the quick-freeze, deep-etch, rotary-replication technique. Methods Cell Biol 22:97–122

Insull W, Jr, Hata Y, Meakin JD, Marchant L, Andrews CW, Buzek B (1974) Morphology of cholesteryl ester-rich inclusions in lesions of atherosclerosis in man. Atheroscler 19:555–560

Karnovsky MJ (1965) A formaldehyde-glutaraldehyde fixative of high oxmolality for use in electron microscopy. J Cell Biol 27:137A

Lang PD, Insull W, Jr (1970) Lipid droplets in atherosclerotic fatty streaks of human aorta. J Clin Invest 49:1479–1488

Luckenbill LM, Cohen AS (1966) The association of lipid droplets with cytoplasmic filaments in avian subsynovial adipose cells. J Cell Biol 31:195–199

Lundberg B (1975) The similarity between mesomorphic droplets in atherosclerotic lesions and cholesteryl ester suspensions. Chem Phys Lipids 14:309–312

Marshall JR, Adams JG, O'Neal RM, de Bakey ME (1966) The ultrastructure of uncomplicated human atheroma in surgically resected aortas. J Atheroscler Res 6:120–131

McGookey DJ, Anderson RGW (1983) Morphological characterization of the cholesteryl ester cycle in cultured mouse macrophage foam cells. J Cell Biol 97:1156–1168

Peters TJ, Takano T, de Duve C (1973) Subcellular fractionation studies on the cells isolated from normal and atherosclerotic aorta. Atherogenesis: Initiating Factors (Ciba Foundation Symposium), Elsevier, Amsterdam, P 197

Portman OW (1970) Arterial composition and metabolism esterified fatty acids and cholesterol. Adv Lipid Res 8:41–114

Ruska H, Ruska C, Meyer-Delpho W (1972) Das Gefrieratzbild von Lipidablagerungen in der Aortenintima cholesteringefutterter Kaninchen. [Lipid droplets in the aorta intima of cholesterol fed rabbits as seen by freeze-etching]. Virchows Arch Abt B Zellpath 11:279–283

Shio H, Farquhar MG, de Duve C (1974) Lysosomes of the arterial wall. IV. Cytochemical localization of acid phosphatase and catalase in smooth muscle cells and foam cells from rabbit atheromatous aorta. Am J Pathol 76:1–16

Shio H, Haley NJ, Fowler S (1979) Characterization of lipid-laden aortic cells from cholesterol-fed rabbits. III. Intracellular localization of cholesterol and cholesteryl ester. Lab Invest 41:160–167

Smith RE, Farquhar MG (1966) Lysosome function in the regulation of the secretory process in cells of the anterior pituitary gland. J Cell Biol 31:319–347

Takano T, Muto K, Imanaka T, Ohkuma S (1981) Lysosomal acid cholesterol esterase in low-density lysosomes from the atheromatous aorta in cholesterol-fed rabbits. Biochem Int 2:229–236

Takano T, Muto K, Imanaka T, Ohkuma S (1982) Preparation of low-density lysosomal membranes from atheromatous aorta of cholesterol-fed rabbits. Biochem Int 4:485–492

Takano T, Amanuma-Muto K, Imanaka T, Ohkuma S (1984) Cinemicrophotographic observation of foam cells containing anisotropic liquid crystals. Acta Histochem Cytochem 17:421–426

Wake K (1974) Development of vitamin A-rich lipid droplets in multivesicular bodies of rat liver stellate cells. J Cell Biol 63:683–691

Watanabe Y (1980) Serial in breeding of rabbits with hereditaryhyperlipidemia (WHHL-Rabbit). Atherosclerosis 36:261–268

Wood EM (1967) An ordered complex of filaments surrounding the lipid droplets in developing adipose cells. Anat Rec 157:437–447

Accepted August 31, 1986