Ultrastructure of the intima in WHHL and cholesterol-fed rabbit aortas prepared by ultra-rapid freezing and freeze-etching

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Abstract Intima from aortas of normal Watanabe Heritable Hyperlipidemic (WHHL) and cholesterol-fed (10 days - 3 months) rabbits were examined by ultra-rapid freezing without chemical fixation followed by rotary shadow freeze-etching. The extracellular matrix in areas devoid of cells was seen in extraordinary detail and consisted of a reticulum of thick filaments, finer branching filaments, collagen fibrils, and granules of varying sizes. No lipid deposits were seen in normal intima. However, the subendothelial region of WHHL intima was filled with collagen fibrils surrounding and entwined between clusters of discrete lipid vesicles that ranged in size from 23 to 169 nm. Approximately 80% of the lipid vesicles in the WHHL rabbit intima measured between 70 and 169 nm. The lipid particles in the WHHL intima always appeared in clusters, many of which appeared to be fusing into larger size vesicles. These aggregates were clearly linked to the matrix filaments. A similar deposition of lipid particles was seen in the extracellular matrix of cholesterol-fed rabbits but in contrast to the particle size distribution of the WHHL intima, more than 75% of the particles in the cholesterol-fed intima had a diameter between 23 and 68 nm and 51% were between 23 and 45 nm. We conclude that in cell-free areas of WHHL and after only 10 days of cholesterol feeding, lipoprotein-derived lipid is present in the intima as clusters of vesicles enmeshed in the complex extracellular matrix.—**Frank, J. S., and A. M. Fogelman.** Ultrastructure of the intima in WHHL and cholesterol-fed rabbit aortas prepared by ultra-rapid freezing and freeze-etching. *J. Lipid Res.* 1989. **30:** 967–978.

Supplementary key words electron microscopy • lipid particles

While lipoproteins are believed to accumulate in the artery wall prior to the migration of monocytes in the pathogenesis of atherosIcerotic lesions (1-3), the mechanism for their retention and the state in which they are retained in the intima of the wall have yet to be determined. Based on immunologic and morphologic observations and attempts to remove lipoproteins from artery wall specimens, it has been presumed that at least some lipid and lipoproteins are intimately associated with the artery wall matrix (4-6). There have been several in vitro studies that have also suggested that lipoprotein-proteoglycan interactions may be important in the early stages of lesion formation (7-10).

Recent ultrastructural studies using conventional freeze-fracture and modified thin-section electron microscopy demonstrated the presence of "extracellular liposomes" (100–300 nm in diameter) within the intima in the second week of a high cholesterol diet (11) and the presence of apolipoprotein B in the intima after only 1 week of a high cholesterol diet (12). Ultrastructural evidence to support a lipid-matrix association and proof that lipid/lipoprotein aggregates actually exist in vivo have been difficult to obtain because of the technical difficulties in preserving the integrity of lipid-rich structures after fixation and processing. In the studies to date (11, 12) it has not been possible to identify particles smaller than 100 nm in the artery wall, suggesting that these techniques might lack the sensitivity to see particles in the range of low density lipoproteins (LDL) and beta-migrating very low density lipoproteins (β-VLDL). Poor retention and staining of lipid as well as the extraction and collapse of proteoglycan with chemical fixation and routine electron microscopy techniques have been a chief deterrent to the detailed ultrastructural description of the components of the extracellular matrix.

In this report we describe the use of ultra-rapid freezing and rotary shadow freeze-etching (13–15) as a method for preserving the fine structure of the extracellular matrix. This method clearly retains the components of the matrix in as close to the in vivo condition as has been possible to

Abbreviations: LDL, low density lipoprotein; VLDL, very low density lipoprotein; β-VLDL, beta migrating VLDL; WHHL, Watanabe Heritable Hyperlipidemic.

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date. We demonstrate that lipid deposits are present in the aortic intima of Watanabe heritable hyperlipidemic (WHHL) and cholesterol-fed rabbits after only 10 days of feeding but not in the intima of normal rabbits. The deposits appear to be interwoven and in direct contact with the extracellular matrix. Some of these lipid deposits were seen to have characteristics strongly suggestive of coalesced lipid/lipoprotein complexes. Moreover, an analysis of particle size distribution indicated a greater percent of larger lipid particles in the aortic intima of WHHL rabbits as compared to cholesterol-fed rabbits.

MATERIAL AND METHODS

Aortic preparation

Normal New Zealand White rabbits (4 and 8 months old) were purchased from commercial sources and maintained on normal rabbit chow. The diet for cholesterol-fed rabbits was prepared by adding 2% cholesterol (w/w) in corn oil (10% w/w) to the normal Purina rabbit chow; the rabbits were fed the diet for 10, 14, and 16 days, and for 3 months. WHHL rabbits (2 and 8 months of age) were obtained from our breeding colony (16). The animals were anesthetized with nembutal and their aortas were rapidly removed and immediately immersed in cold buffered saline. Sections of tissue (3 mm^2) were cut from the aortic arch and upper thoracic aorta. The sections chosen did not have any visible lesions. The sections of aorta were immediately placed on slabs of gelatin resting on aluminum support discs. The disc was shaped to fit on the cold stage of the Balzers 301 freeze-fracture apparatus and the freezing head of the Polarion quick-freeze apparatus (17). The freeing head with gelatin slab and adhered piece of aorta was plunged onto an ultrapure copper block that had been precooled to 4°K by liquid helium as previously described (14, 15).

Freeze-fracture and replication

Each frozen piece of aorta was fractured very superficially (max. between 7 and 20 μm) to insure that the fracture plane was within the intima of the vessel wall. It was determined that one pass of the microtome arm produced fractures that were 80% within endothelial cells and two or three passes of the knife produced a fracture plane in the intima. Fracturing was performed at -120°C and a vacuum of 1×10^{-7} Torr. For deep-etching the specimen stage was warmed to -90°C and maintained at this temperature for approximately 8 min. The fractured and etched surface of the aorta was rotary replicated. This involved depositing 2 nm of platinum/carbon from the electron-beam gun mounted at a 25° angle while the tissue rotated through 360° six times. This was followed by three short (5 to 8 sec) bursts of carbon (14). The fractured and etched replica was digested overnight in Purex bleach, washed in distilled water, and picked up on 3-mm specimen grids.

Quantitation

The data on the extracellular matrix filaments and the lipid particle size were collected from negatives taken of random areas of the intima from control (two animals), WHHL (two animals), and cholesterol-fed (10 days, one animal; 2 weeks, two animals; 3 months, one animal) rabbits. Ten micrographs from ten different areas of intima were examined per animal. Within these areas 130 and 100 lipid particle diameters were measured in WHHL and cholesterol-fed aortas, respectively. The negatives photographed at an original magnification of 20,000 were projected onto the screen of a Nikon Profile projector for measurement.

Electron microscopy

The replicas were examined at 80 kV in a JEOL 100 CX electron microscope. The negatives were printed as negative images.

RESULTS

Control animals

The intima of the normal rabbit aorta was seen to consist of endothelial cells, basal lamina, and the subendothelial matrix containing collagen fibers, elastin microfibrils, and proteoglycans (data not shown). When the aorta was ultra-rapidly frozen without chemical fixation, fractured, and etched, the subendothelial matrix components were seen in extraordinary detail (**Fig. 1**). The area shown in Fig. 1 was beneath the endothelial cell layer and consists of collagen fibrils linked together with an extensive network of filaments presumed to be proteoglycans. In 20 replicas examined from two control aortas the matrix was free of extracellular lipid inclusions. The micrographs in Fig. 1 and **Fig. 2** reveal a heterogeneous network consisting of a reticulum of thick filaments, finer branching filaments, collagen fibrils, and granules of varying sizes. It was not possible to assess the length of the filaments due to the tremendous variability in this parameter. The thickness of the filaments uncorrected for platinum varied between 6.7 and 7.9 nm for the thick filaments and 2.2 nm to 2.3 nm for the finer filaments. The collagen fibrils were readily apparent and etching of the unfixed collagen revealed the presence of periodically spaced (67.4 nm) transverse cuffs of material on the surface of the collagen which no doubt contributes to the characteristic crossbanding of the fibril. Frequently, the junction between the thick filaments and the collagen fibrils was via a granule which appeared to be very prominent along the collagen fibril in these freeze-

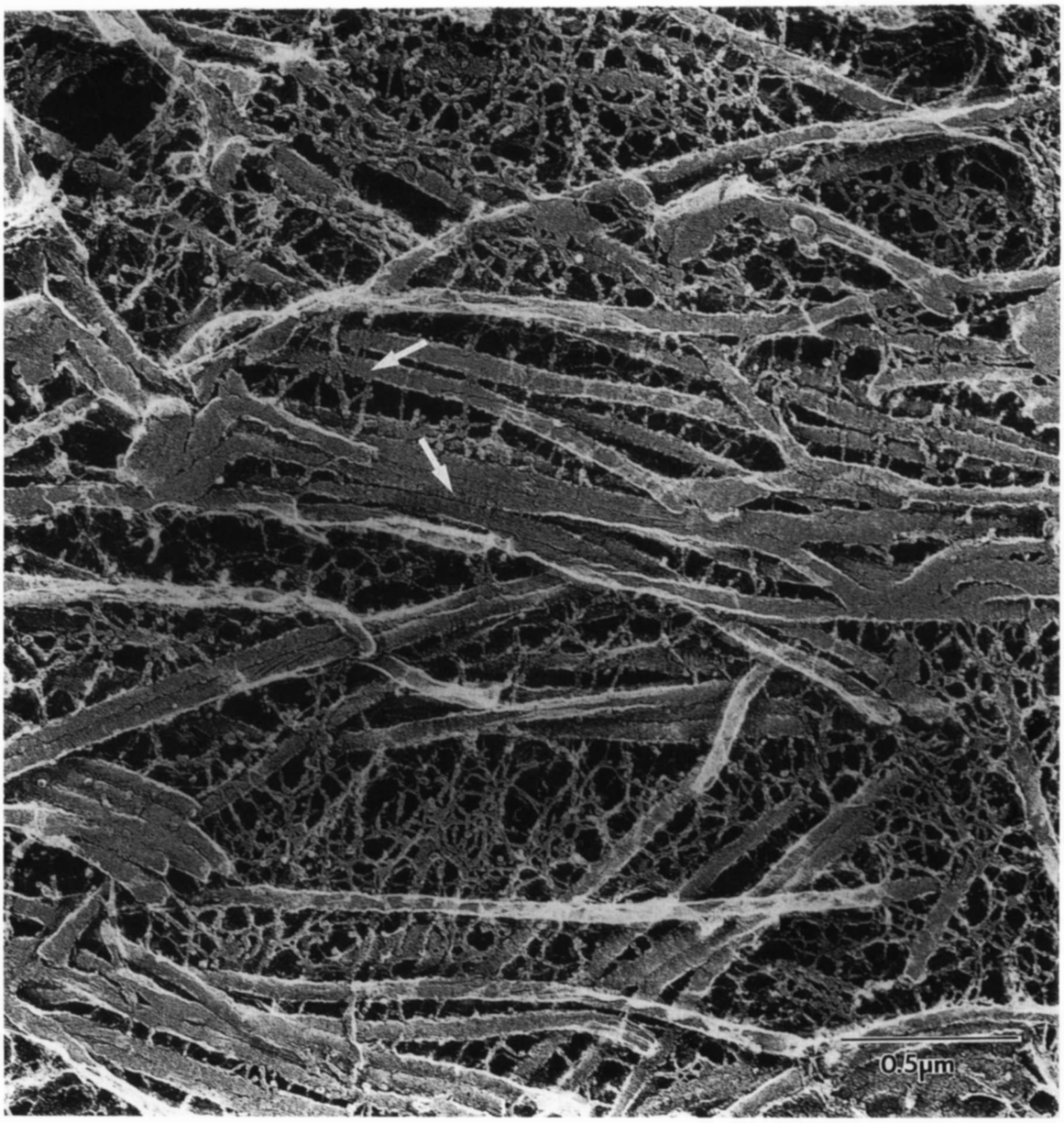

Fig. 1. Portion of the intima just under an endothelial cell from a control rabbit aorta at the region of the arch. Collagen fibrils (arrows) are clearly evident in a matrix of filaments. ×, 50,625.

etch preparations (Fig. 2). The micrographs suggest that these granules are a prevalent component of the extracellular matrix network. However, the variation in size of the granules raises the possibility that some granules may be formed as a result of disruption of the thick or finer branching filaments during etching.

Examination of Figs. 1 and 2 reveals the presence of numerous contact sites between the collagen fibrils and the thick and finer branching filaments. Both types of filaments were seen to branch extensively. The thick filament appeared to form contact sites on the collagen fibrils. Although less frequent, contact sites were also seen between the finer branching filaments and the collagen fibrils.

Experimental animals

WHHL rabbits. **Fig. 3** demonstrates that the subendothelial region of the WHHL intima was filled with columns of collagen fibrils surrounding and entwined between clusters of lipid vesicles ranging in size between 23 nm and 169 nm. Fig. 3 also demonstrates the extensive connections between the filaments of the matrix, the lipid particles, and

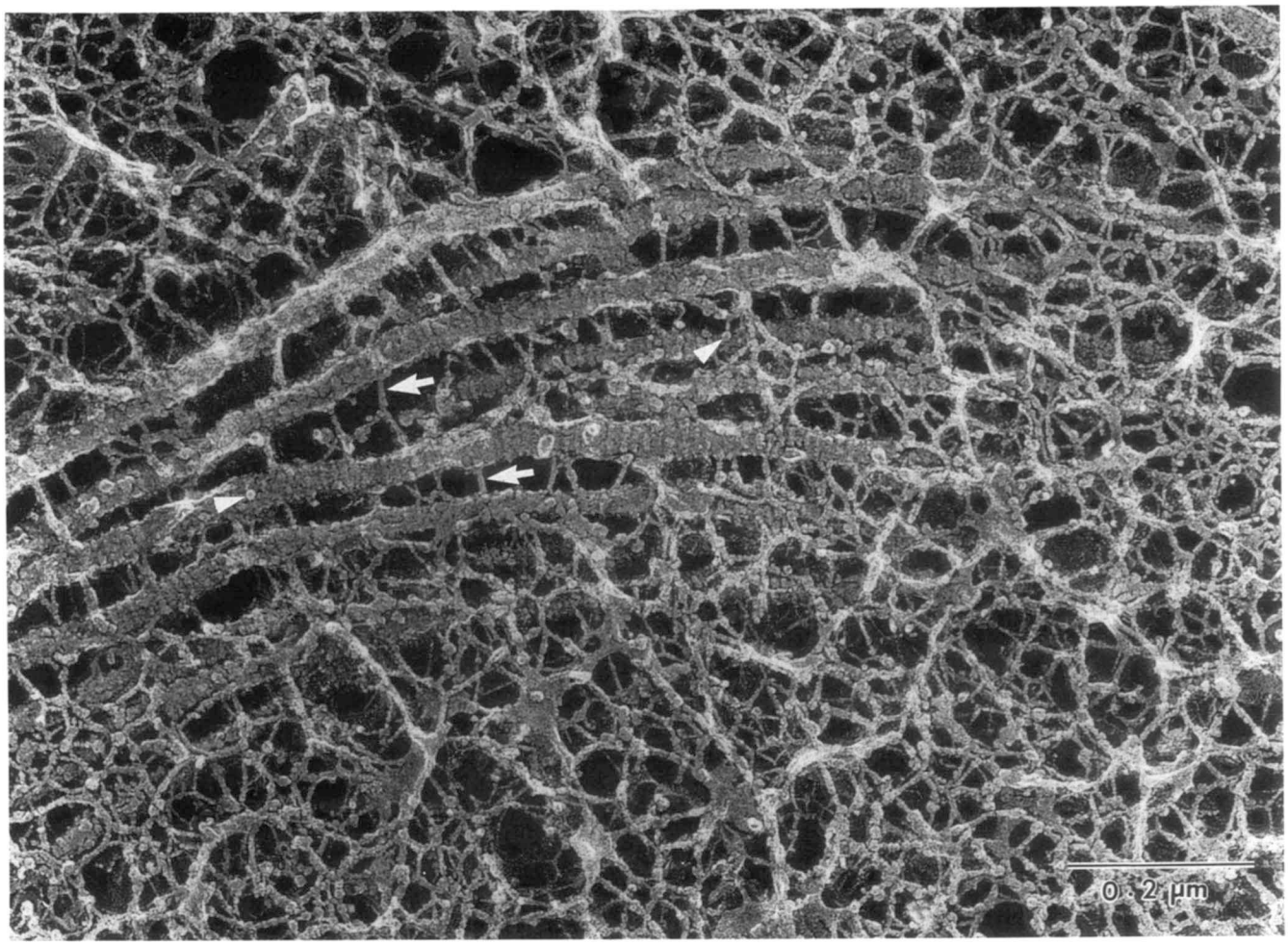

Fig. 2. Higher magnification micrograph from area similar to Fig. 1 from a control rabbit. Collagen fibrils occupy the center of the micrograph. Periodic cross-banding characteristic of these fibrils is evident. Periodically along the collagen fibrils short filaments connect fibrils to each other and to the network of the extracellular matrix (arrows). Granules of varying sizes are also present at junctions of the matrix filaments and also along the length of the collagen fibrils (arrowhead). ×, 126,360.

the collagen fibrils. While lipid particles as small as 23 nm could be resolved, the majority (80%) of the particles in the WHHL rabbits measured between 70 and 169 nm (**Fig. 4**). The lipid particles in the WHHL specimens always appeared in clusters and many appeared to be fusing into larger size vesicles. In all of the replicas studied the coalesced vesicles were never seen to be free of the matrix but always appeared to be linked to the matrix proteoglycan. Collagen generally surrounded these structures and was frequently seen to be in direct contact with the liposomes (Fig. 3, inset).

Cholesterol-fed rabbits. Typical areas from the intima of a rabbit fed cholesterol for only 10 days are seen in **Fig. 5** and **Fig. 6** and for the 16-day cholesterol-fed animals in **Fig. 7** and **Fig. 8.** In all replicas examined clusters of lipid vesicles were present in the intima. The lipid clusters appeared to be held in position by direct contact with filaments of the matrix and with collagen fibrils. It is striking that even in the earliest period of cholesterol feeding (10 days) clusters of lipid consisting of vesicles of varying diameters were seen. Occasionally individual, small diameter (41 nm) lipid vesicles were seen adjacent to small clusters of vesicles just below the endothelial cells (Fig. 6). By 16 days larger aggregates were present (Fig. 7) at a time when cellular infiltration into the intima was not yet present. Not unexpectedly, aortic segments from the 3-month cholesterol-fed rabbits contained vast areas filled with aggregates of lipid. It is important to stress that all areas of intima examined in these studies appeared to be free of cells or cellular debris. Even in the 3-month cholesterol-fed animal only areas without macroscopic lesions were chosen and only areas free of cells and cellular debris were studied. Fig. 8 is a micrograph that demonstrates the same type of connections between the filamentous matrix, the collagen fibrils, and the lipid vesicles as was shown in the WHHL intima (Fig. 3).

The size of the lipid vesicles in the intima of the cholesterol-fed animals was strikingly smaller than that seen in

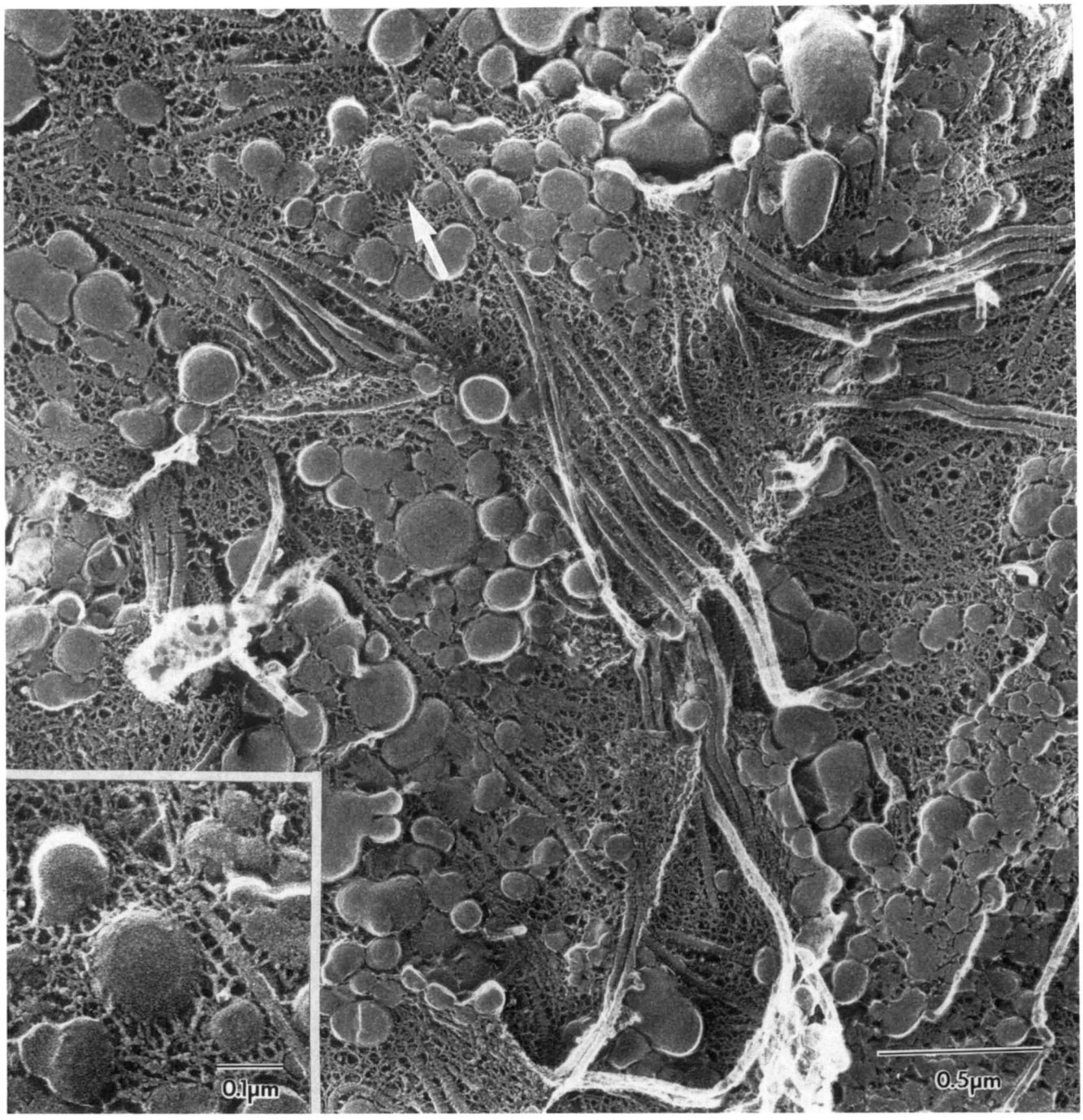

Fig. 3. Portion of the intima from WHHL rabbit aortic arch. The intima contains lipid vesicles enmeshed in the filament network that appears to link vesicles to each other and to numerous collagen fibrils. Arrow points to the direct attachment of matrix filaments to the surface of a lipid vesicle. Inset shows this in higher magnification. ×, 53,136: inset ×, 108,000.

the WHHL rabbits (Fig. 4). More than 75% of the lipid vesicles in the intima of the cholesterol-fed rabbits had a diameter between 20 and 68 nm, and 51% were between 23 and 45 nm. In contrast, 80% of the lipid vesicle diameters in the intima of the WHHL rabbits were between 70 and 169 nm.

DISCUSSION

In the present study we have demonstrated the ability to visualize in a quasi-three-dimensional way the intricate ultrastructure of matrix components and the lipid-like particles in WHHL and cholesterol-fed rabbit aortic in-

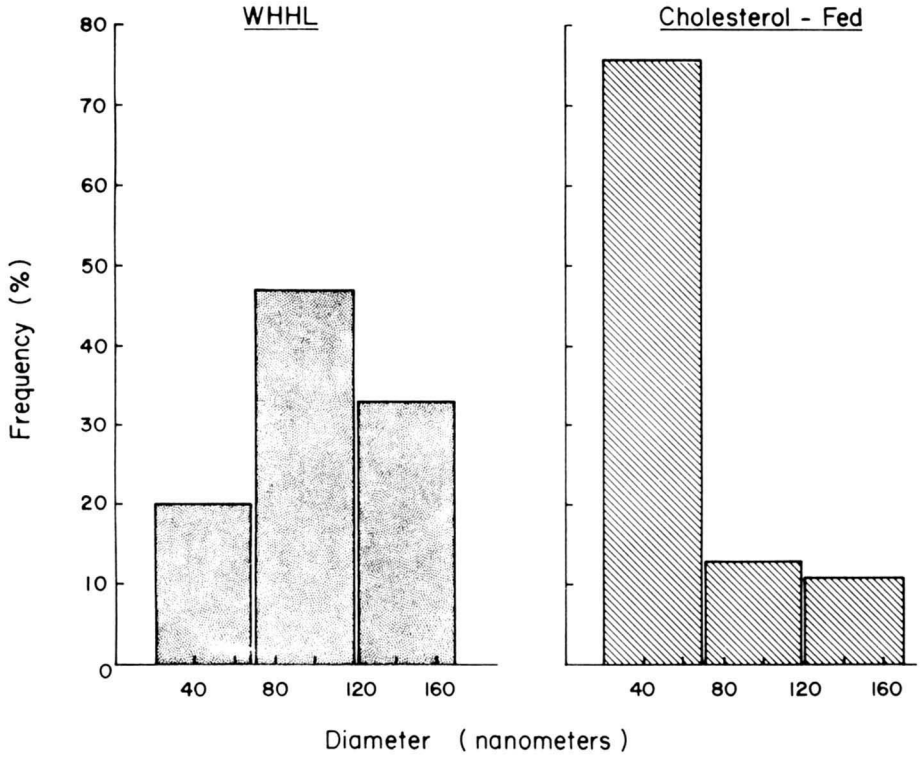

Fig. 4. Histograms showing the lipid particle diameter frequency in WHHL and cholesterol-fed animals measured from photographic prints. All lipid particles measured were in the intima. In the cholesterol-fed group, data from 10- and 16-day cholesterol-fed animals were combined.

tima. In tissue prepared with ultra-rapid freezing without chemical treatment followed by freeze etching we found: *1*) that the lipid-rich vesicles present in the intima in areas devoid of cells or cell debris were contained within a network of filaments forming the extracellular matrix; *2*) the matrix filaments appeared to directly contact individual lipid particles as well as larger aggregates of lipid; *3*) the lipid appeared in clusters after only 10 days of cholesterol feeding; *4*) most of the lipid vesicles appeared to have a smooth surface; *5*) the size distribution of the vesicles was easily determined because of the exceptional preservation. The vesicles ranged from 23 to 169 nm; 80% of the particles in WHHL rabbits were between 70 and 169 nm; more than 75% of the lipid vesicles in the intima of the cholesterol-fed rabbits had a diameter between 23 and 68 nm.

The difference in diameter betwen lipid particles in WHHL and cholesterol-fed rabbits is striking. Based on their size and their concentration in WHHL plasma (18, 19), the lipid particles in the WHHL intima could be aggregates of LDL or triglyceride-rich remnants. The electron micrographs suggest that fusion of vesicles was occurring. This is supported by studies that showed that the aortic permeability coefficients (intimal clearances) of plasma macromolecules decrease linearly as the logarithm of the diameter of the molecule increases (20, 21). It appears that macromolecules with diameters larger than 75 nm have negligible aortic permeability (21). Since 75% of the lipid vesicles within the intima of the WHHL rabbits had a diameter of 75–169 nm, vesicle fusion within the aggregates is very likely to have occurred. The lipid particles present in the matrix of both the 10- and 16-day cholesterol-fed animals had diameters within the range of β-VLDL or LDL. It is striking that in the 10-day cholesterol-fed animal, in addition to aggregates of lipid vesicles, individual vesicles between 30 and 40 nm appeared just below the endothelial cells enmeshed in the filaments of the basement membrane-matrix complex (Fig. 6). It is in this same area of the rabbit aorta that Mora, Lupu, and Simionescu (12) demonstrated the presence of apolipoprotein B after 1 week of cholesterol feeding.

The idea that extracellular elements in the matrix (i.e., proteoglycans, collagen, and elastin), could play an important role in lipid and lipoprotein deposition in the artery wall has been actively investigated for a number of years (20). Early histochemical studies showed a close spatial relationship between the metachromatic regions (presumably containing mucopolysaccarides) and those regions that took up lipid stain (22, 23). In vitro studies have suggested that glycosaminoglycans can form complexes with human plasma lipoproteins (24, 25) and other studies

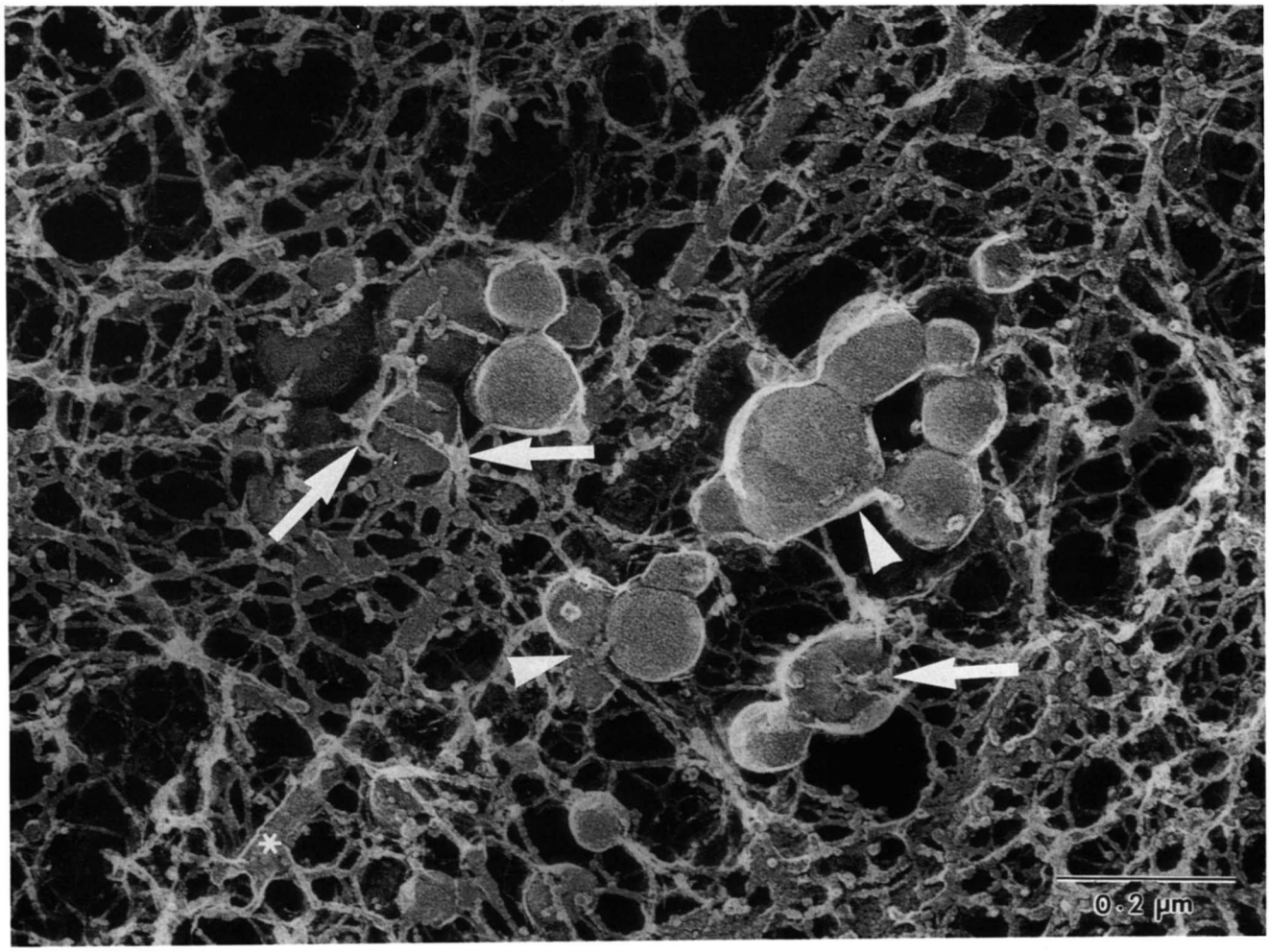

Fig. 5. Portion of the intima from a 10-day cholesterol-fed rabbit. Clusters of lipid vesicles (arrowheads) are held in position by filaments of the matrix (arrows). A strand of what is likely collagen (asterisk) runs between the two clusters of lipid vesicles and is linked via matrix filaments to the lipid. ×, 126,360.

have shown that VLDL and LDL can bind to arterial glycosaminoglycans at physiological pH and ionic strength (26). Woodward et al. (27) isolated and partially characterized intact lipoprotein–glycosaminoglycan complexes from human plaques. The implication has been that proteoglycan or glycosaminoglycan within the subendothelial matrix traps lipid and/or lipoproteins. The work of Wight and Ross (28) and Wight (29) first suggested a three-dimensional net of proteoglycan and interconnecting collagen fibrils based on thin-sections stained with ruthenium red. A striking feature of the images in Figs. 1, 2, and 3 is the extensive cage-like structure of the matrix filaments and the extent of their interactions with collagen. These images support the possibility that complex macromolecules such as lipoproteins could have multiple interactions if caught in this three-dimensional meshwork. Wolfbauer et al. (30) demonstrated in vitro that cholesteryl ester-rich lipid droplets from macrophages could be taken up by smooth muscle cells if the lipid droplets are allowed to come in contact with the smooth muscle cells.

The subsequent fate of particles trapped in the extracellular matrix may be determined as a result of an alteration of their surface charge and finally by their receptor recognition. Studies with a chondroitin-6-SO_4-rich proteoglycan-LDL aggregate isolated from rabbit and human intima indicate that the specificity and affinity of the association depends on the intact structure of the proteoglycan and the modulated surface charge as well as the lipid composition of the apoB lipoproteins (31). Following the demonstration by Goldstein et al. that acetyl-LDL could cause massive cholesteryl ester accumulation in macrophages (32), Fogelman et al. (33) demonstrated that LDL modified in vitro by malondialdehyde produced a similar accumulation of cholesteryl ester in macrophages and Fogelman et al. (33) postulated that malondialdehyde produced by lipid peroxidation may modify LDL in the

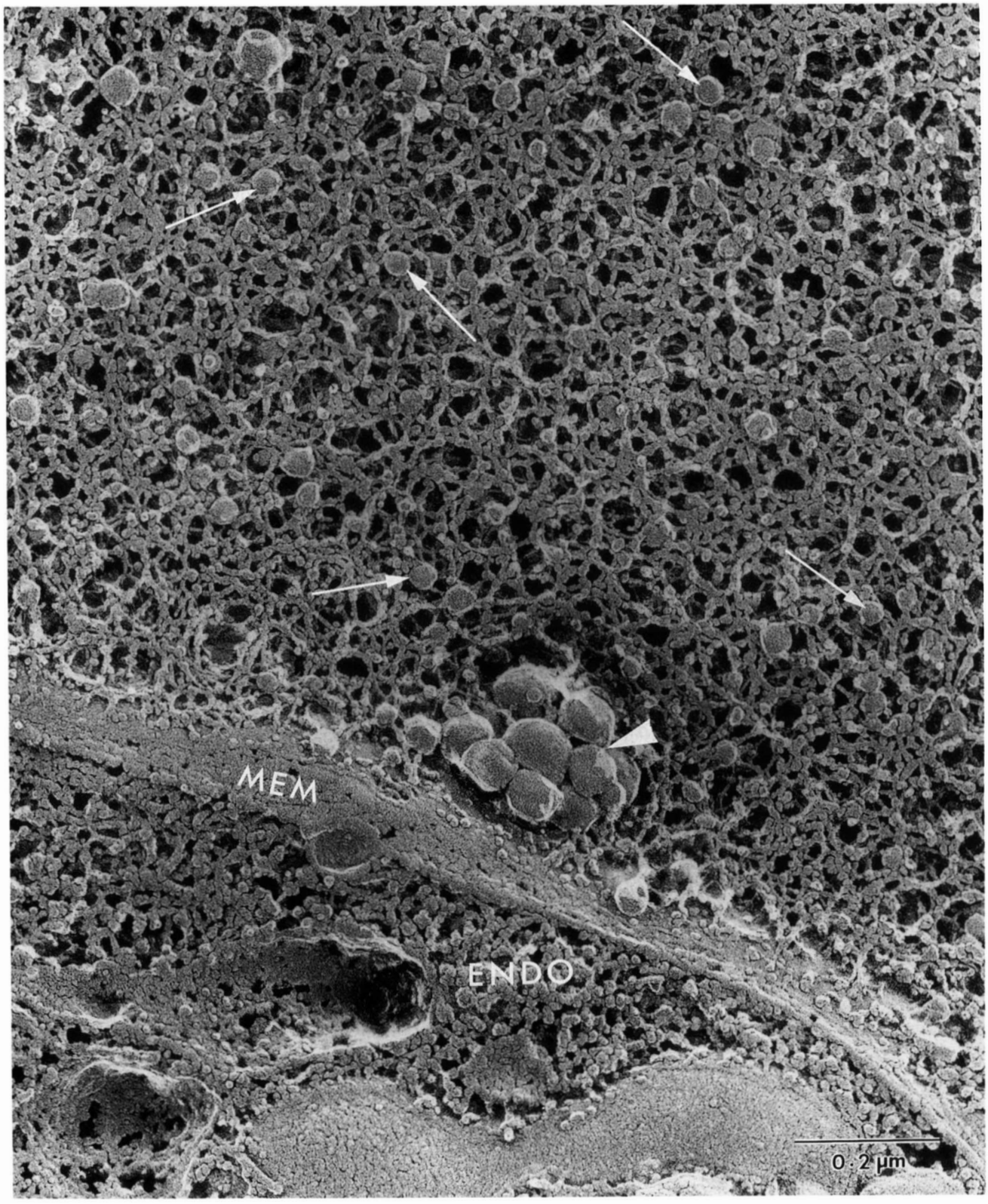

Fig. 6. This micrograph is from a 10-day cholesterol-fed rabbit. In the bottom of the micrography is a portion of an endothelial cell (ENDO), while in the middle and top portion of the figure the basal lamina matrix of the endothelial cell is seen.The portion of the endothelial cell facing the lumen of the aorta, although not seen here, is at the bottom of the figure. Etching has exposed a portion of the endothelial membrane (MEM) facing the intima. Arrowhead points to a cluster of lipid vesicles just below the cell membrane. Notice the extensive basal lamina filaments and the presence of small diameter lipid vesicles within this matrix (arrows). ×, 126,360.

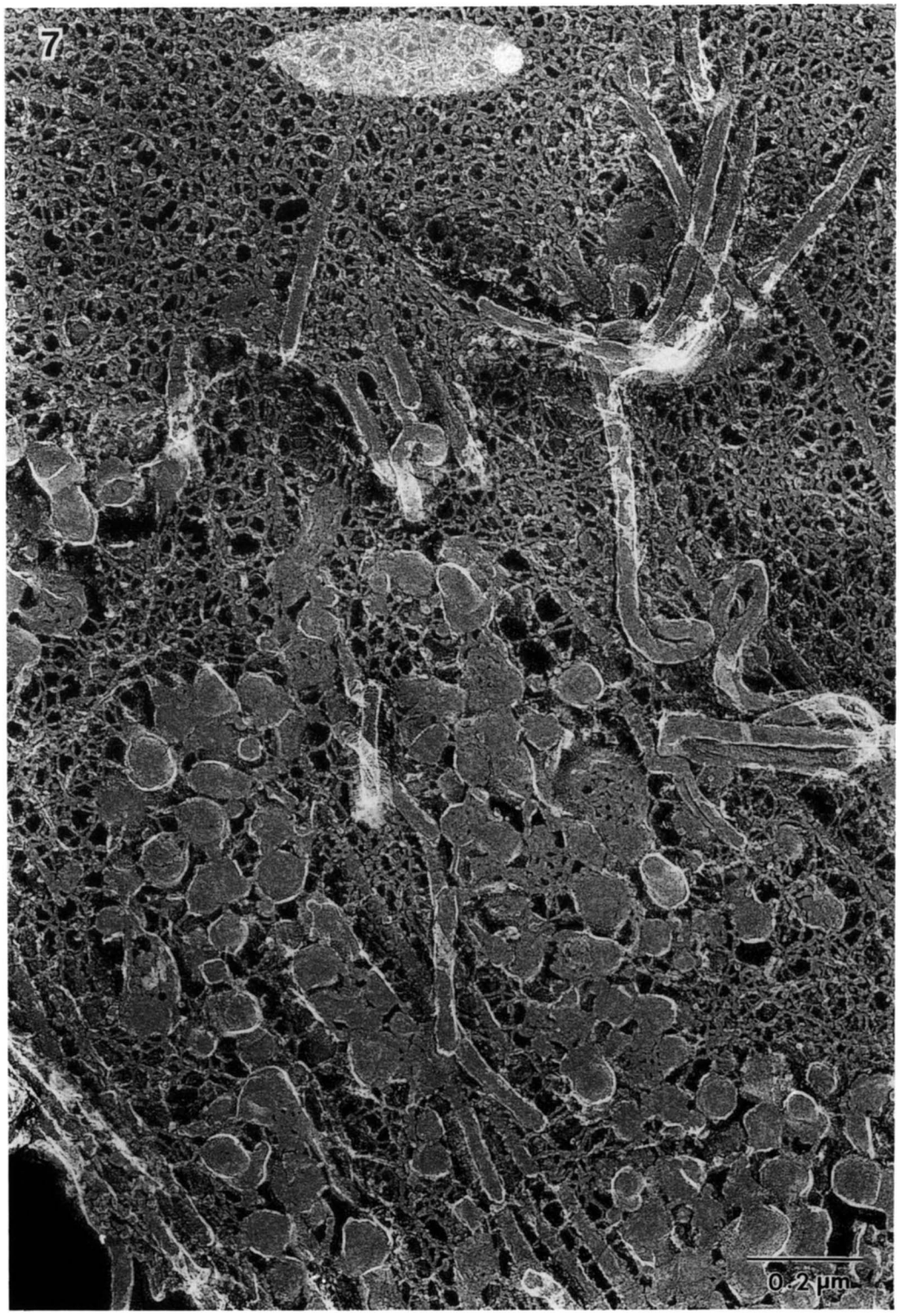

Fig. 7. Portion of the intima from a 16-day cholesterol-fed rabbit. Large aggregates of lipid vesicles are surrounded and enmeshed in extracellular matrix and collagen fibrisl. ×, 86,184.

artery wall. Subsequently, Haberland, Fogelman, and Edwards (34) demonstrated that modification of LDL bound to glycosaminoglycan by malondialdehyde led to release of the lipoprotein and a change in recognition from the LDL receptor to the scavenger receptor. Havel et al. (35) have shown that, in addition to accumulating LDL,

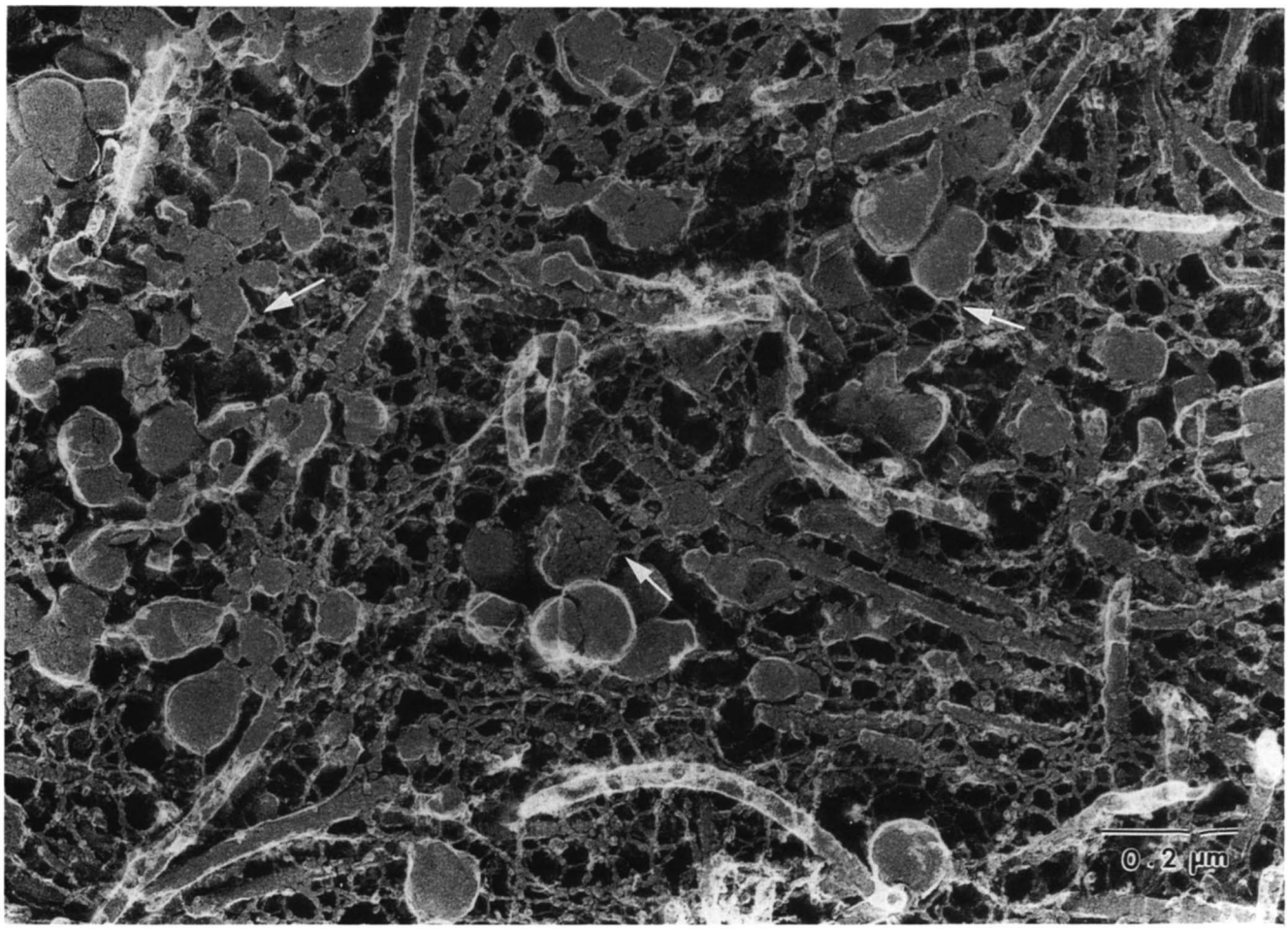

Fig. 8. Higher magnification micrograph from a 16-day cholesterol-fed animal. The connections between various size lipid vesicles with filaments and collagen fibrils are easily seen. ×, 126,360.

WHHL rabbits also accumulate triglyceride-rich lipoproteins such as VLDL. In this regard it is interesting to note that VLDL can also be modified and subsequently recognized by the scavenger receptor (36). The experiments of Haberland, Fong, and Cheng (37) indicate that lipoproteins are in fact modified in vivo in the artery walls of WHHL rabbits by products of lipid peroxidation, specifically malondialdehyde. Fogelman et al. (33) also demonstrated in 1980 that cross-linking LDL molecules with glutaraldehyde produced macromolecular aggregates of LDL that were taken up by human monocyte-macrophages and produced massive cholesteryl ester accumulation. Recent experiments by Hoff et al. (38) have demonstrated that 4-hydroxynonenal, an aldehyde which is produced as a result of lipid peroxidation in vivo, can cross-link LDL and produce aggregates that cause massive cholesteryl ester accumulation upon being ingested by macrophages. Other evidence that lipoprotein aggregates can cause cholesteryl ester accumulation in macrophages comes from the work of Suits, Heinecke, and Chait (39). They demonstrated that LDL aggregates formed in vitro can bind to the surface of macrophages via the LDL receptor and subsequently be ingested by phagocytosis causing massive cholesteryl ester formation. Khoo et al. (40) recently demonstrated enhanced macrophage uptake of LDL aggregates formed by simply vortexing the LDL. They stressed the ease with which LDL can self-aggregate. As the data in this study demonstrate, the lipid vesicles within rabbit intima occurred as aggregates after only 10 days of cholesterol feeding.

Simionescu et al. (11) identified unesterified, cholesterol-rich lipid particles in the intima of rabbits that were cholesterol-fed for only 2 weeks. During this period β-VLDL–gold complexes were found in endothelial plasmalemmal vesicles and in the subendothelium space. This same laboratory recently demonstrated the presence of apoB lipoproteins in subendothelial space after one week of a high fat diet (12). The size of their "extracellular liposomes" was larger (range 100–300 nm) than those reported here for cholesterol-fed animals. The differences

may in part be due to the greater ability of the techniques described in this report to retain and to resolve the smaller size particles in unfixed ultra-rapidly frozen tissue. Indeed, the type of preparative technique may be critical in determining the accurate size distribution of lipid particles. We think it unlikely that the lipid particles demonstrated in our study were derived from cells. However, our data clearly demonstrate that lipoproteins and/or lipid vesicles are held in place in the intima of WHHL and cholesterol-fed rabbits in such a way that cells entering these areas are likely to come in direct contact with them.

The techniques demonstrated in this report represent a markedly improved ultrastructural method for monitoring the events in the subendothelial space. Future studies aimed at identification of both the type of lipid particles and their specific proteoglycan attachment will be important to a better understanding of the pathogenesis of atherosclerosis.

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