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# Early Extracellular and Cellular Lipid Deposits in Aorta of Cholesterol-fed Rabbits

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***Subendothelial accumulation of extracellular liposomes rich in unesterified cholesterol has been described as an early feature of atherosclerosis induced by cholesterol feeding in rabbits. Beta-very-low-density lipoproteins, however, the presumed source of atherogenic lipid in this animal model, contain mostly esterified cholesterol. The purpose of this study was to test for the presence of extracellular neutral lipid deposits consistent with esterified cholesterol, by employing new electron microscopic techniques. Rabbits were fed 0.5% cholesterol, 5% butter for 0, 1, 2, and 4 weeks. The lipid-preserving ultrastructural techniques showed, in control and atherosclerotic rabbit arteries, neutral lipid droplets adherent to the endothelial luminal surface. After 1 to 2 weeks, subendothelial extracellular deposits of mostly membranous lipid appeared; these deposits contained variable amounts of neutral lipid. At the same time, cytoplasmic neutral lipid droplets appeared in smooth muscle cells and in a small number of subendothelial macrophagelike cells. After 4 weeks, monocytic infiltration and macrophage foam cell development were prominent, but abundant extracellular lipid deposits also were found. Therefore, in arteries of cholesterol-fed rabbits, deposition of membranous and neutral lipid in the extracellular space and neutral lipid accumulation in resident arterial cells are early and probably independent events, both occurring before monocytic infiltration of the arterial intima. (Am J Pathol 1992, 141:925–936)***

Lipid deposits in the human atherosclerotic fibrous plaque are predominantly extracellular. Considerable evidence suggests that most of this lipid is deposited directly in the extracellular space without prior accumulation in foam cells.[1–4] Efforts to elucidate mechanisms of extracellular lipid deposition have been hampered, however, by the fact that animal atherosclerosis generally exhibits mostly cellular lipid and by the fact that extracellular lipid is poorly preserved by routine electron microscopic processing.[5] Several investigators recently have described extracellular lipid deposits in animal atherosclerosis. Kruth found extracellular lipid in cholesterol-fed rabbits, rats, monkeys, and swine.[6–8] In particular, subendothelial accumulation of cholesterol-rich particles stained with the fluorescent probe filipin was observed soon after the onset of cholesterol feeding in rabbits.[8] Simionescu et al[9,10] used electron microscopy and immunocytochemistry to demonstrate the subendothelial accumulation of extracellular phospholipid liposomes rich in unesterified cholesterol, along with apolipoprotein B, in rabbit aorta within the first 2 weeks of cholesterol feeding, before monocyte diapedesis. By freeze-etching electron microscopy, Frank and Fogelman[11] acquired images consistent with the presence of extracellular lipoproteins and fusion particles in rabbit atherosclerosis.

The electron microscopic techniques used in these previous studies did not allow the identification of neutral lipid representing cholesteryl ester deposits. Because beta-very-low-density lipoproteins (β-VLDL), the presumed source of lipid deposits, contain mostly neutral lipid in the form of cholesteryl ester, it is important to determine whether neutral lipid is present in early rabbit atherogenesis along with membranous (liposomal) lipid. The current study has used new electron microscopic techniques[5] to preserve neutral as well as membranous lipid in rabbit aorta after 1, 2, and 4 weeks of cholesterol feeding. This includes the period before the infiltration of monocytes into the arterial intima. To provide comparison with previous work, a 5% butter, 0.5% cholesterol diet was employed.[9,10]

## Materials and Methods

### Animal Model

Normal New Zealand White rabbits weighing 3 to 4 lbs. were obtained from Hop-Stop (Houston, TX) and re-

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mained healthy throughout the experimental protocol. An atherogenic diet was prepared by Teklad Diets (Madison, WI) by adding 0.5% cholesterol, 5% unsalted butter to routine rabbit chow (Teklad #0533). Four rabbits were killed after each of three intervals—1, 2, and 4 weeks—of feeding the atherogenic diet; four control rabbits were killed after 1 to 6 weeks on the same diet without added cholesterol or fat. Plasma was obtained from the middle ear artery at baseline and after 3 days, 1, 2, and 4 weeks for lipoprotein analysis in the Atherosclerosis Clinical Laboratory of The Methodist Hospital, Houston, Texas.

Rabbits were anesthetized with an intramuscular injection of ketamine 32 mg/kg, xylazine 6.4 mg/kg and acepromazine 1.05 mg/kg. The left carotid artery was cannulated and infused at a pressure of 110 mm Hg with 125 ml Hank's balanced salt solution, followed by 1000 ml 1% paraformaldehyde, 1.25% glutaraldehyde in 0.13 mol/l (molar) phosphate buffer, pH 7.4,[12] over a period of 15 minutes. The left femoral artery was sectioned distal to the inguinal ligament to allow outflow of blood and perfusates at a rate controlled by pressure applied to the site. The entire aorta and initial portions of innominate, left carotid, and iliac arteries were removed and further fixed overnight in 2% paraformaldehyde, 2.5% glutaraldehyde in 0.13 mol/l Na phosphate buffer, pH 7.4. Two rinses each of 0.13 mol/l Na phosphate buffer, pH 7.4, were made.

## *Tissue Dissection*

Tissue blocks were selected at eight sites from each aortic specimen—proximal aortic arch, arch plus proximal innominate, arch plus proximal left axillary and carotid, distal arch, midthoracic aorta, midabdominal aorta including celiac orifice, and left and right iliacs. Nearly all significant lipid deposition, even after 4 weeks, was confined to the aortic arch. Branch points in particular were susceptible to both extracellular and cellular lipid deposits, with the greatest accumulation of lipid occurring at the lateral aortic aspect of branches such as the innominate and left carotid. For comparison with vascular lipid deposition, a portion of the Achilles tendon was also dissected and processed.

## *Cytochemical Procedures*

Blocks of arterial tissue were rinsed twice in 0.1 mol/l Na cacodylate buffer, pH 7.4, and then processed for electron microscopy as described previously[5] by both the osmium-thiocarbohydrazide-osmium (OTO) and osmium-tannic acid-paraphenylenediamine (TA-PDA, alternatively designated as OTAP) techniques. The OTO procedure involved an initial treatment of the tissue with cacodylate-buffered 2% $OsO_4$, extensive washing, treatment with 1.5% aqueous thiocarbahydrazide (Sigma, St. Louis, MO), washing, and a second osmication. The OTAP procedure used osmication followed by mordanting with 1% buffered tannic acid (Fisher Scientific, Pittsburgh, PA), then a wash in buffered 1% $NaSO_4$. During the initial stages of dehydration in the OTAP procedure, tissues were exposed to 1% paraphenylenediamine (Sigma) in 70% ethanol. For both procedures, dehydration schedules proceeded through 70%, 95%, and 100% ethanol within 45 minutes to a 1:1 mixture of LX112 resin (Ladd Research Industries, Burlington, VT) and ethanol for 1 hour, then pure resin overnight. On the following day, two changes of LX112 resin were made, and then the tissue was embedded in LX112 resin.

Thin sections were cut at 70 to 90 nm using an LKB Ultrotome III. The OTO-prepared specimens were viewed without further staining, and OTAP specimens underwent section staining with 7% uranyl acetate in 50% ethanol and then Sato's lead citrate.[13] Electron microscopy was performed with a JEOL 200CX microscope operating at 80 kV.

All tissue sites at the various times were examined systematically with micrographs obtained at locations where lipid appeared. In addition, for the four control rabbits and the four rabbits fed the atherogenic diet for 2 weeks, six micrographs were obtained in random fashion, without regard to the presence or absence of lipid deposits, at each of three sites—adjacent to innominate orifice, adjacent to left carotid orifice, and distal arch.

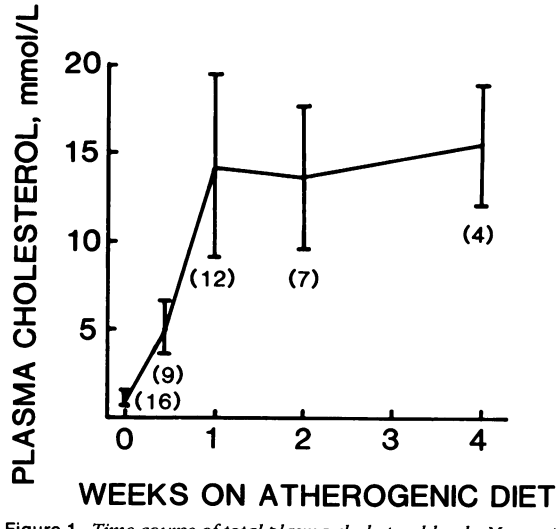


**Figure 1.** *Time course of total plasma cholesterol levels. Means ± standard deviations are shown. Shown in parentheses are the numbers of rabbits studied at each time period. To obtain results in mg/dl, multiply by 38.7.*

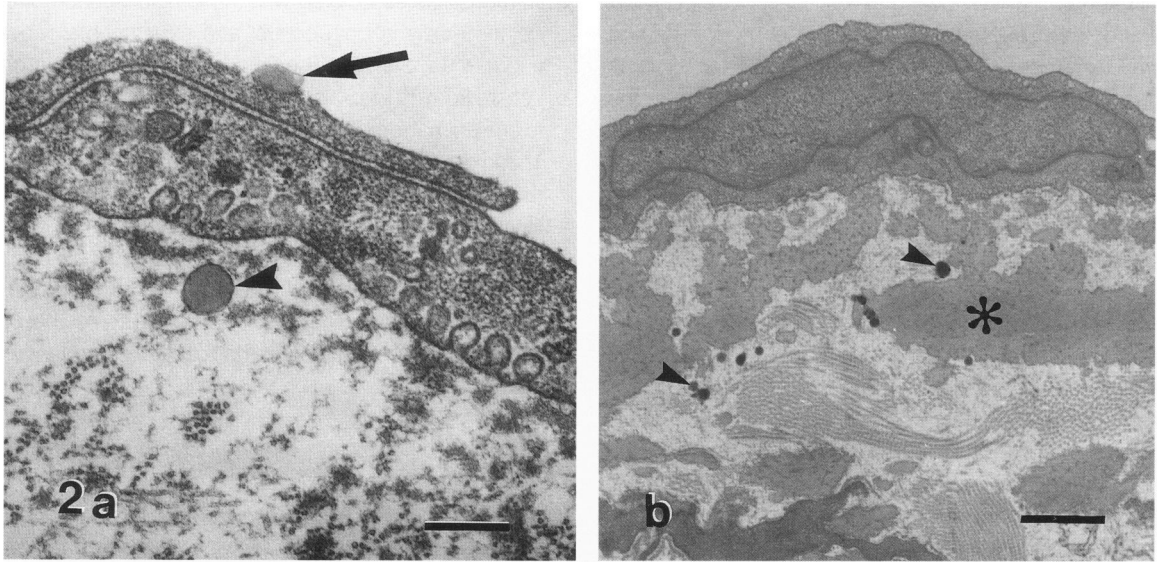


**Figure 2.** *Lipid deposits in control rabbits.* **a:** *Neutral lipid droplet (arrow) adhering to the luminal surface of the aortic endothelium at the site of the innominate branch. Neutral lipid droplets (arrowhead) in the subendothelial space were occasionally observed, and they usually exhibited smooth contours. OTAP procedure, ×54,000, bar = 200 nm.* **b:** *Smooth, round lipid droplets (arrowheads) mostly associated with elastic fibers (asterisk) in the distal aortic arch. By OTO processing, shown here, lipid deposits are generally the most electron dense objects in the microscopic field. ×10,800, bar = 1 μm.*

## Results

### Plasma Lipid Analyses

Figure 1 shows that plasma total cholesterol levels reached a plateau after 1 week on the atherogenic diet. High-density lipoprotein cholesterol levels rose slightly from 0.8 ± 0.3 mmol/L (mean ± standard deviation [SD]) to an average of 1.4 ± 0.4 mmol/l in weeks 1 through of the experimental diet. Triglyceride levels also rose modestly from 0.8 ± 0.3 to 1.2 ± 0.8 mmol/L.

### Electron Microscopy

#### Control Animals

The general ultrastructural findings in control rabbits were consistent with previous descriptions.[14,15] A narrow

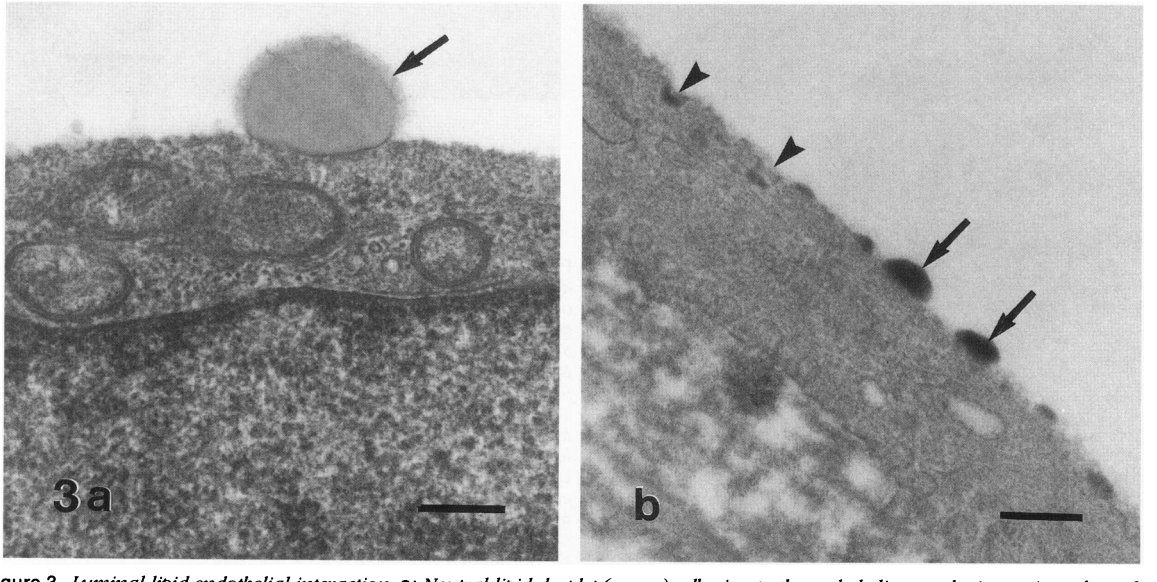


**Figure 3.** *Luminal lipid-endothelial interaction.* **a:** *Neutral lipid droplet (arrow) adhering to the endothelium at the innominate branch of the aortic arch. One week of cholesterol feeding, OTAP cytochemistry, ×57,800, bar = 200 nm.* **b:** *Lipid can be observed as β-VLDL-sized particles on the endothelium surface (arrows) and possibly as dark-staining material lining pits forming pinocytic vesicles (arrowheads). Micrograph was taken in the distal aortic arch. One week of cholesterol feeding, OTO cytochemistry, ×53,300, bar = 200 nm.*

intimal layer was composed almost solely of a monolayer of endothelial cells resting on basement membrane and a layer of elastin, which was usually fragmented. Smooth muscle cells, whether residing in intimal pads or in the tunica media, were mostly separated from the endothelial cells by an elastic lamina and were of the contractile phenotype with abundant myofilaments. Occasional mononuclear cells resembling macrophages were found immediately beneath the endothelium.

A new finding, ascribed to lipid-preserving electron microscopic techniques, was the appearance of round neutral lipid droplets in the arterial intima and inner media (Figures 2a and b). Most of the droplets were adjacent to elastic fibers. Each of four control rabbits exhibited this form of lipid deposition, as did several rabbits fed the atherogenic diet. There was no apparent increase in this particular type of lipid deposit in the experimental rabbits.

Another new finding was the appearance of neutral lipid droplets associated with the luminal surface of endothelial cells in both control and experimental rabbits (Figures 2a, 3a, 3b). The lipid droplets ranged in size usually from 50 to 150 nm and occasionally up to 500 nm. In one animal, droplets up to 5000 nm in size were found. These droplets were too common (found in one fourth of randomly positioned micrographs) to be ascribed to artifact, although an artifactual derivation of some of the largest droplets cannot be ruled out. As a rough approximation, one droplet was found per 50 $\mu^2$ of luminal surface area. These luminal surface droplets, somewhat surprisingly, did not increase significantly in frequency in fat-fed rabbits.

### *One Week of Atherogenic Diet*

The ultrastructural appearance of the rabbit aorta after 1 week of the atherogenic diet was mostly normal, even

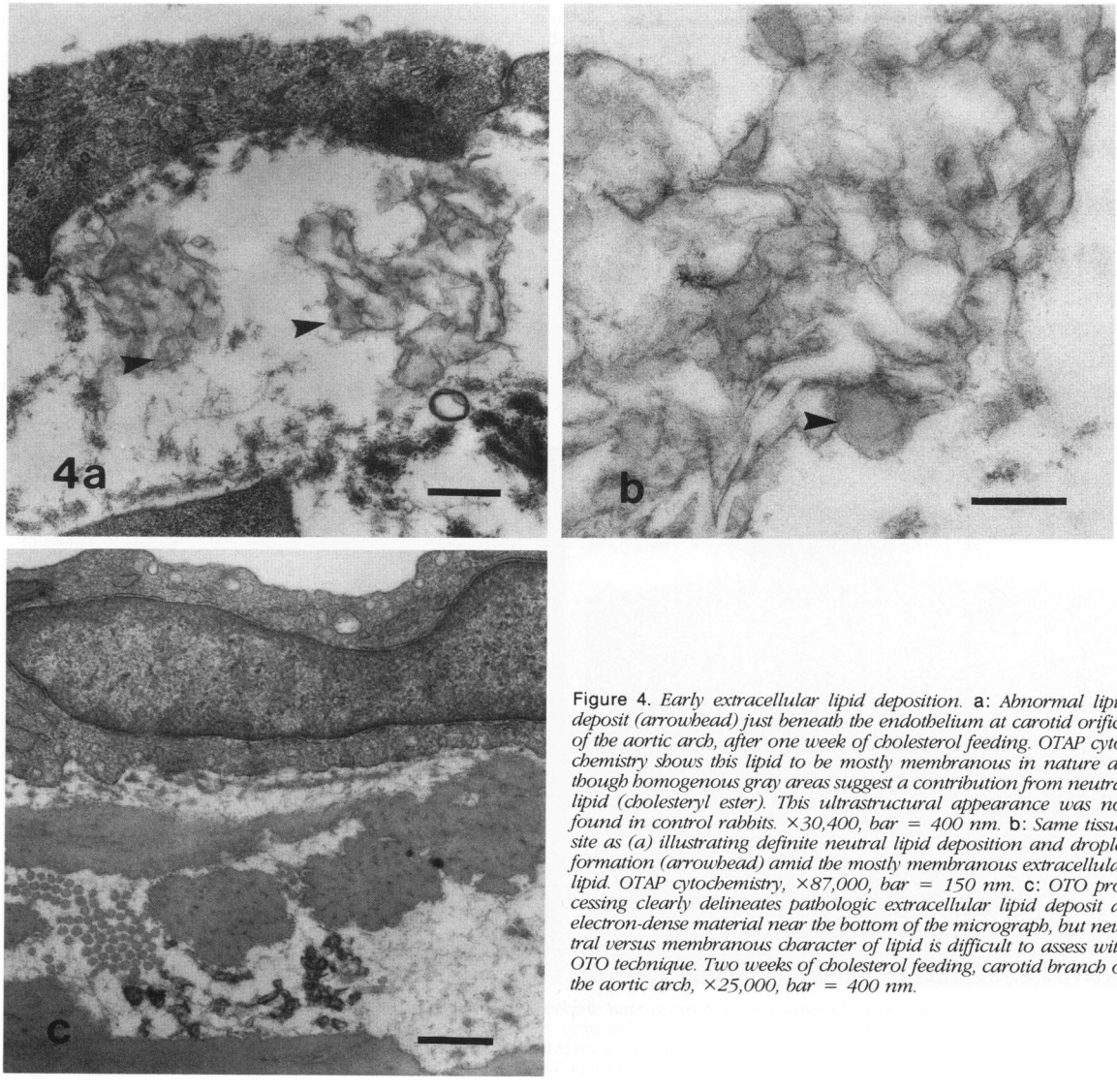


Figure 4. *Early extracellular lipid deposition.* a: *Abnormal lipid deposit (arrowhead) just beneath the endothelium at carotid orifice of the aortic arch, after one week of cholesterol feeding. OTAP cytochemistry shows this lipid to be mostly membranous in nature although homogenous gray areas suggest a contribution from neutral lipid (cholesteryl ester). This ultrastructural appearance was not found in control rabbits. ×30,400, bar = 400 nm.* b: *Same tissue site as (a) illustrating definite neutral lipid deposition and droplet formation (arrowhead) amid the mostly membranous extracellular lipid. OTAP cytochemistry, ×87,000, bar = 150 nm.* c: *OTO processing clearly delineates pathologic extracellular lipid deposit as electron-dense material near the bottom of the micrograph, but neutral versus membranous character of lipid is difficult to assess with OTO technique. Two weeks of cholesterol feeding, carotid branch of the aortic arch, ×25,000, bar = 400 nm.*

at sites of atherosclerotic predilection. Nevertheless, distinctly abnormal findings occurred in each of the four rabbits studied at this time. Cytoplasmic lipid droplets were found with increased frequency in the resident cells of the inner aortic wall—smooth muscle cells, endothelial cells, and occasional macrophages. There was no evidence of abnormal monocytic infiltration of the subendothelial space at this time.

An abnormal structure in the subendothelial extracellular space, observed only in experimental animals, was a deposit of mostly membranous lipid with irregular contours, often extending a micrometer in overall size (Figure 4a, b, c). Most such structures contained a lesser amount of neutral lipid, identified by OTAP staining, in addition to membranous lipid. These structures were distinctly different from the isolated, spherical neutral lipid droplets described above in control rabbits.

### *Two Weeks of Atherogenic Diet*

More extensive deposits of mostly membranous lipid were found at this time in subendothelial locations and deeper in the aortic wall (Figures 4c, 5). The deposits were multicentric and interspersed with connective tissue elements. No transitional forms were identified to provide evidence that the extracellular lipid deposits originated from cell death or from gemmation of cellular processes.

An increased number of smooth muscle cells, endothelial cells, and macrophages contained cytoplasmic lipid droplets at 2 weeks. This time period marked the beginning of monocyte diapedesis across the endothelial layer; Figure 5 shows an adherent mononuclear leukocyte. Occasional isolated subendothelial foam cells with abundant lipid accumulation were found. However, the number of non–smooth muscle foam cells and macrophages in experimental rabbit aortas appeared no greater than the number of macrophages residing in control aortic subendothelium.

An assessment of the prevalence of various forms of lipid deposition at this stage of atherogenesis was performed by examining 72 experimental and 72 control randomly positioned micrographs of OTO-stained aortic tissue. Thirteen of these micrographs showed abnormal extracellular lipid deposition. Fifteen showed cytoplasmic lipid droplets in smooth muscle cells identified by the presence of myofilaments, peripheral dense attachment sites, and surrounding basement membrane; and four micrographs revealed abundant lipid accumulation in subendothelial cells (possibly macrophages) lacking the features of contractile smooth muscle cells. In contrast, only two micrographs from control rabbits showed minimal cellular lipid deposits, which occurred in smooth muscle cells.

### *Four Weeks of Atherogenic Diet*

At this time both cellular and extracellular lipid deposition in the inner arterial wall were well established (Fig-

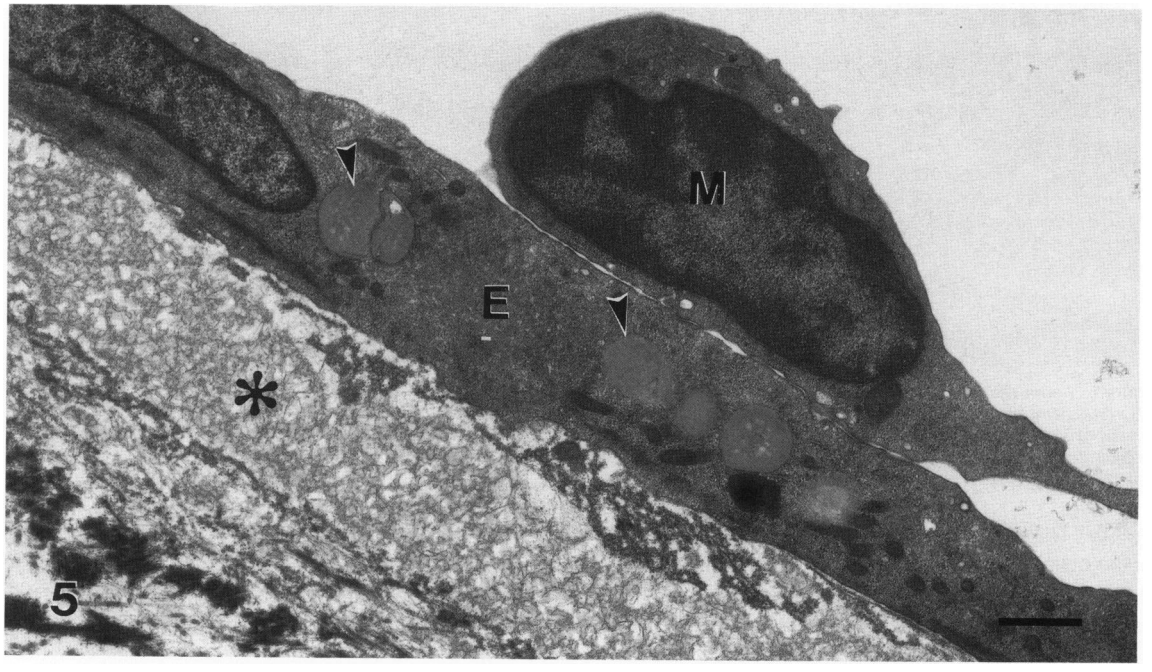


**Figure 5.** *Extensive extracellular lipid deposition at 2 weeks of feeding. The subendothelial space is widened by a band of lipid deposits (asterisk), which contain both membranous and neutral lipid demonstrated by the OTAP procedure. The endothelial cell (E) has accumulated several lipid droplets (arrowheads). Adherence of mononuclear leukocyte (M) signals an early phase of monocytic infiltration of the arterial intimal. Aortic arch at left carotid orifice, OTAP cytochemistry, ×10,900, bar = 1 μm.*

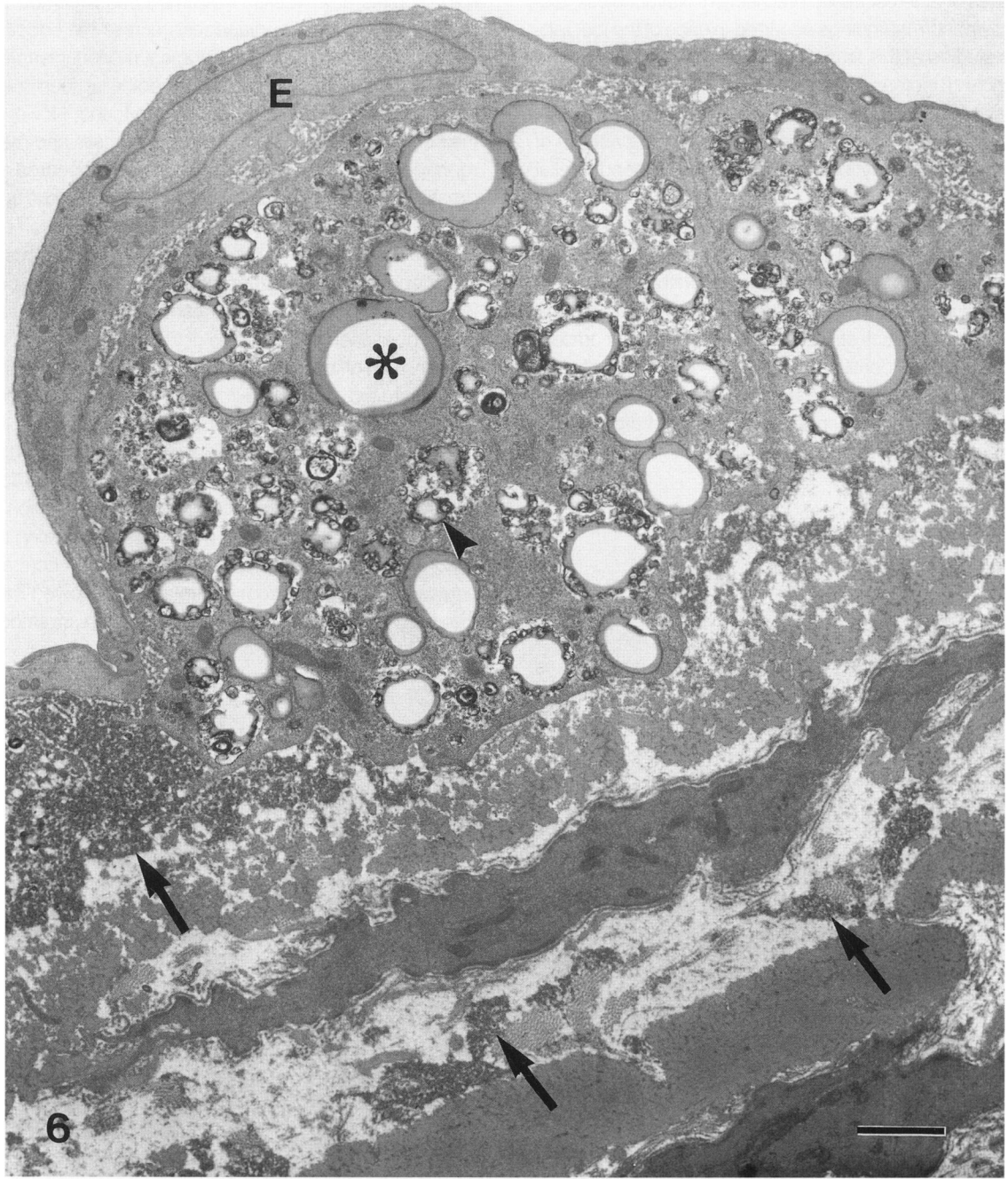


**Figure 6.** *Foam cell formation in a subendothelial area which also exhibits extracellular lipid, demonstrated as electron dense material by OTO cytochemistry (arrows). The foam cell has distended the endothelium (E) resulting in fatty streak formation at the intimal pad of the carotid orifice, a location prone to lesion development. The foam cell contains both smooth cytoplasmic lipid droplets (asterisk), as well as droplets that are probably lysosomal (arrowhead). Note the morphologic distinction between the cellular and extracellular lipid. Intimal pad of carotid orifice, ×8,800, bar = 1.5 μm.*

ures 6–11). Cellular lipid in foam cells clearly accounted for the bulk of total arterial lipid deposition (Figures 6 and 7). Foam cells were sometimes surrounded by extracellular lipid deposits, but in other instances foam cells occurred in a setting devoid of visible extracellular lipid. The presence of large secondary lysosomes in subendothelial foam cells was demonstrated by acid phosphatase cytochemistry (Figure 7). Attempts to combine acid phosphatase cytochemistry with OTO or OTAP cytochemistry were unsuccessful. Nevertheless, it seems likely that cellular lipid droplets with ragged, pitted edges in Figure 6 reside within lysosomes.

Figure 6 shows extracellular lipid deposits in proximity to a subendothelial foam cell. The ultrastructural appear-

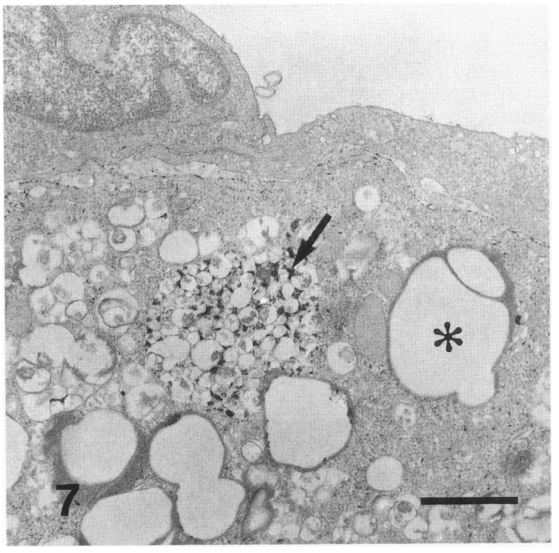


**Figure 7.** *Acid phosphatase cytochemistry at four weeks of diet. A foam cell residing just beneath the endothelium exhibits characteristic lead staining within a large secondary lysosome (arrow). Smooth contoured cytoplasmic lipid droplets (asterisk) are unstained. Lipid has been mostly extracted from the tissue due to lack of OTO or OTAP lipid-preserving techniques.* ×*16,000, bar = 800 μm.*

ance of extracellular lipid—densely packed, uniformly small electron dense deposits by OTO technique—is quite distinct from the heterogeneous, more vesicular appearance of lipid-filled lysosomes and from the smooth contours of large cytoplasmic lipid droplets. The distinctive ultrastructure of extracellular lipid deposits casts doubt on the hypothesis that they are derived from previously existing cellular lipid accumulations. Moreover, Figure 6 shows extracellular lipid deposits of similar appearance in a subendothelial location and at scattered sites deep to an elastic lamina and a smooth muscle cell. This is an example of the multicentric character of extracellular lipid deposits.

Extracellular lipid deposition was difficult to visualize by light microscopy, but was found to be widespread by electron microscopy. The most striking deposits were found at the margins of foam cell atherosclerotic lesions (Figure 8). Some specimens, however, showed extensive extracellular lipid deposition in areas with very little cellular lipid. Where this extensive lipid deposition occurred, the extracellular matrix, especially the loose proteoglycan structure lying between cells and matrix fibers, was profoundly altered by the infiltration of membranous and neutral lipid. Closely packed structures of dimensions similar to those of β-VLDL (30 to 80 nm) could be seen in some instances (Figure 9). It should be noted, however, that neither OTO nor OTAP as currently employed allows one to confirm a pseudomicellar or lipoproteinlike ultrastructure in such small particles.

Most of the extracellular lipid deposits in atherosclerotic rabbits had an ultrastructural appearance suggesting a substantial membranous lipid component with small droplets of neutral lipid interspersed. Thus, Figures 4a through b and 5 (OTAP) and Figures 4c, 6, and 8 (OTO) appear to be alternative cytochemical representations of the same phenomenon. The pattern varies from one site to another mostly in terms of the extent of tissue infiltration and the density of packing of the lipid. A less common variation is shown in Figure 10, in which relatively large deposits of neutral lipid are seen in the extracellular space, surrounded by much smaller vesicles and granular material. Figure 9 also shows large neutral lipid deposits in a subendothelial location. Rarely seen, but nevertheless of interest because their similarity to human atherosclerosis [4,33], were multilamellar vesicles (Figure 11) deep within a thickened intima after four weeks of fat feeding.

### *Achilles Tendon*

Only scant abnormal lipid deposits were found in the Achilles tendon even after 4 weeks of the atherogenic diet, and these deposits were almost entirely cellular.

## *Discussion*

The primary new finding of this study is that an atherogenic diet fed to rabbits leads rapidly to deposition of neutral lipid (cholesteryl ester) as well as membranous lipid (phospholipid and unesterified cholesterol) in the extracellular space in the aortic arch. Other new information relates to the presence of neutral lipid droplets in two locations in arteries of both control (chow-fed) and experimental rabbits. These locations are at the endothelial luminal surface and adjacent to elastic fibers.

## *Luminal Surface Lipid Droplets*

In control rabbits, the lipid droplets adherent to the endothelial luminal surface presumably represented VLDL and chylomicrons, because these lipoproteins are known to interact with lipoprotein lipase at the endothelial surface. Therefore, the major lipid in the droplets was probably triglyceride. Cytochemical distinction between triglyceride and cholesteryl ester in the droplets is technically difficult and was considered beyond the scope of the current study. Chylomicrons adherent to the luminal surface of capillary endothelium in rat mammary glands have been visualized previously by electron microscopy.[16] The current study is the first to find such structures adherent to arterial endothelium, a fact attributable to the special cytochemical techniques employed.[5] The

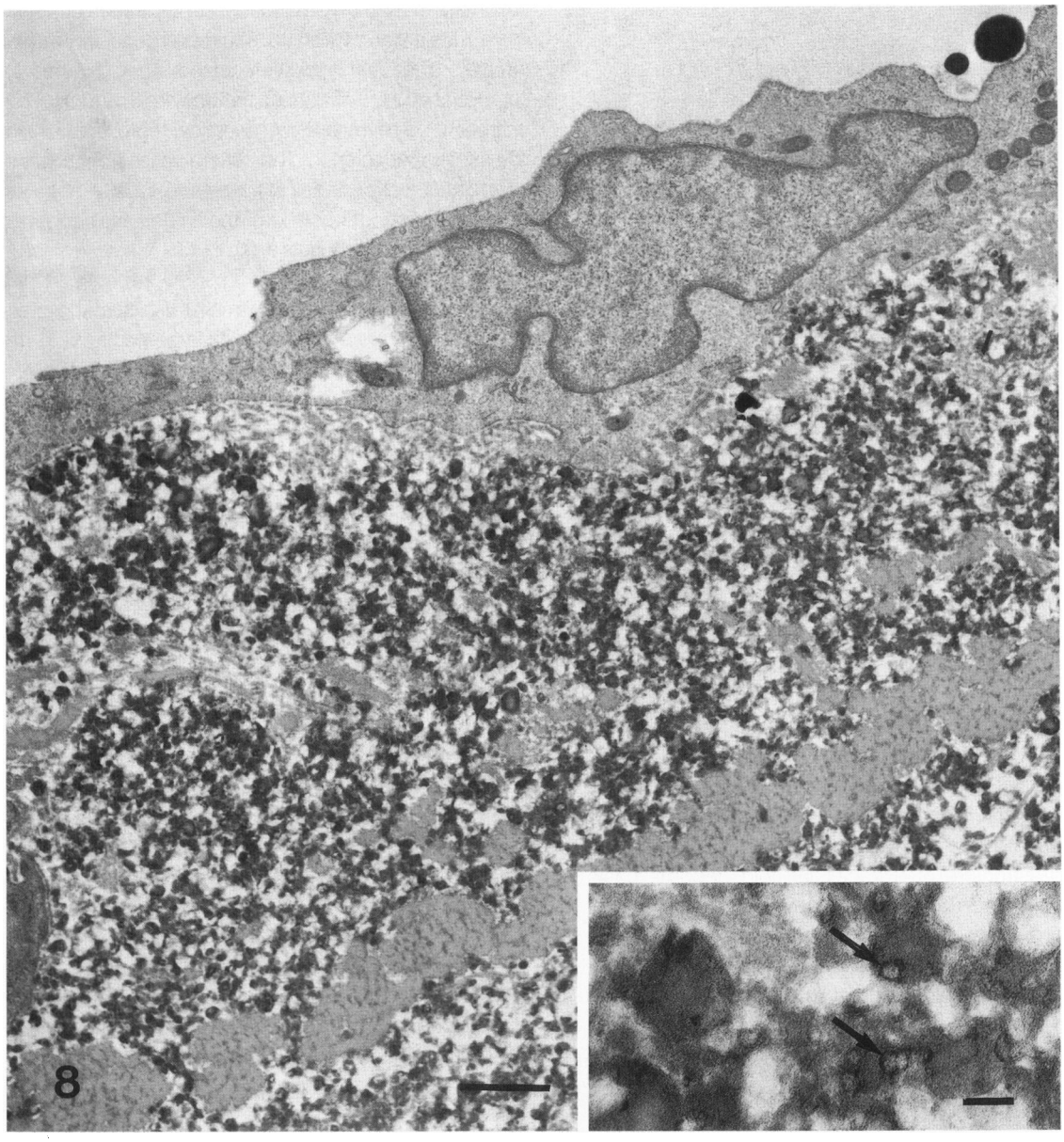


**Figure 8.** *Lipid deposition at four weeks near periphery of foam cell lesion. Extracellular lipid fills available space in the intima and superficial media. OTO cytochemistry demonstrates lipid as electron dense material, but does not delineate membranous lipid clearly. The inset shows abundant of neutral lipid droplets with typical surface pits (arrows). Micrograph taken at a site in the distal aortic arch. ×12,900, bar = 1 μm. Inset: ×69,600, bar = 100 nm.*

large sizes of some luminal surface lipid droplets could be due to lipoprotein fusion occurring *in vivo* or possibly to artifactual fusion during fixation.[11,17] The luminal surface droplets were common structures in control aortas, but surprisingly they did not increase appreciably in frequency in cholesterol-fed rabbits. The β-VLDL which attained massive concentrations in the plasma of cholesterol-fed rabbits may adhere relatively poorly to arterial endothelium, compared with normal VLDL and chylomicrons in chow-fed animals. Lipoprotein lipase, the luminal surface enzyme that interacts with VLDL and chylomicrons, hydrolyzes triglyceride but not cholesteryl esters. Thus it is not unreasonable to propose that cholesteryl ester-rich lipoproteins (β-VLDL) may have relatively weak affinity for lipoprotein lipase and thus relatively weak adherence to arterial endothelium. This proposal requires experimental confirmation. The entire phenomenon of lipoprotein adherence to the arterial luminal surface deserves further study, because postprandial VLDL and chylomicrons have been postulated to deliver cholesterol to atherosclerotic arteries through a lipoprotein lipase–dependent mechanism.[18]

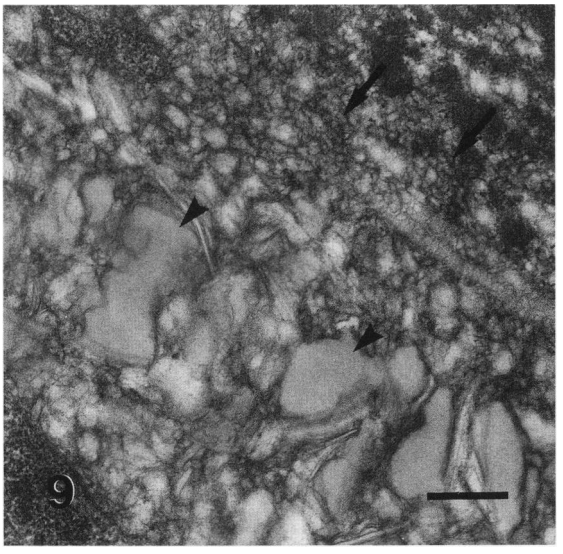

**Figure 9.** *Intense extracellular lipid deposition in a subendothelial location. The smallest structures (arrows) approximate the dimensions of plasma lipoproteins and appear very tightly packed in the extracellular space. Larger deposits of neutral lipid (arrowheads) are also present. Tissue site is the distal aortic arch. Four weeks of diet, OTAP cytochemistry, ×55,200, bar = 200 nm.*

## *Intimal and Medial Lipid Droplets in Control Rabbits*

The neutral lipid droplets adjacent to elastic fibers in normal rabbit arterial wall are somewhat similar in appearance to extracellular droplets found adjacent to and within elastic fibers in the deeper region of human arterial intima.[3] Considerable evidence suggests that the latter droplets are composed mostly of cholesteryl ester,[3] but there is no basis for assuming cholesteryl ester-rich versus triglyceride-rich lipid composition in the case of the droplets found in rabbits. Interestingly, these spherical, isolated droplets did not increase in frequency in the cholesterol-fed rabbits.

## *Early Effects of Diet*

Our observations concur with published results suggesting that infiltration of monocytes into the arterial intima of cholesterol-fed rabbits begins about 2 weeks after diet initiation.[9,19–21] In our random set of micrographs from 2-week diet-fed animals, 15 instances of lipid accumulation occurred in smooth muscle cells exhibiting myofilaments, peripheral dense bodies, and basement membranes, whereas only four instances of lipid accumulation occurred in subendothelial cells lacking these features—possibly macrophages. The latter finding was consistent with the frequency at which similar cells without lipid appeared in normal arterial intima in control rabbits. Therefore, we consider cellular lipid deposition at this time to occur in resident arterial cells, including smooth muscle cells, endothelial cells, and macrophages. Cholesteryl ester accumulation in vascular smooth muscle cells has been enigmatic, because downregulation of LDL receptors ordinarily protects these cells from cholesteryl ester accumulation.[22] Wolfbauer et al.[23] proposed the following sequence of events: lipid droplets are formed initially in arterial macrophages, droplets reach the extracellular space by cell death or other means, and then the macrophage-derived droplets are phagocytosed by smooth muscle cells. The current observations, however, suggest that, at least in the early period after cholesterol feeding, smooth muscle cells can accumulate lipid directly without preliminary uptake in macrophages, because smooth muscle lipid droplets were relatively common despite a paucity of lipid-laden macrophages. Recently, Pitas[24] showed scavenger receptor function in three rabbit smooth muscle cell lines, with upregulation of receptors in the presence of serum.

Abnormal deposits of membranous and neutral lipid in the extracellular space were found as early as 1 week after cholesterol feeding. At 2 weeks, the prevalence of extracellular lipid was slightly less than that of cellular lipid deposits. Unfortunately, the ultrastructural morphology does not lend itself to a quantitative estimate of relative masses of lipid contained in cellular versus extracellular deposits. The notion that extracellular lipid deposition at these early time periods was dependent on prior cellular lipid accumulation seems unlikely, particularly because the extracellular deposits were in some cases widely dispersed and interspersed among connective tissue fibers.

Mora et al.[10,25] found immunoreactive apolipoprotein B co-localizing with extracellular lipid deposits at 2 and 4 weeks, consistent with extracellular derivation of the deposits. Specifically, immunoreactive apolipoprotein B was associated with "extracellular liposomes" by both light and electron microscopy. Liposomes correspond to membranous lipid deposits, but the ultrastructural techniques employed by these authors would not be expected to demonstrate neutral lipid, even if it were present in their material. Thus, they specifically identified the protein component of β-VLDL, the presumed source of extracellular lipid deposits, but did not demonstrate ultrastructurally cholesteryl ester, the major lipid component of β-VLDL.

Additional evidence of lipoprotein involvement in early rabbit atherosclerosis comes from radiolabeled tracer studies of Schwenke and Carew,[20] who found focal accumulation of LDL at lesion-susceptible sites in rabbits fed cholesterol for 8 to 16 days, before the appearance of substantial numbers of macrophage foam cells.[20]

The current results demonstrate the presence of neu-

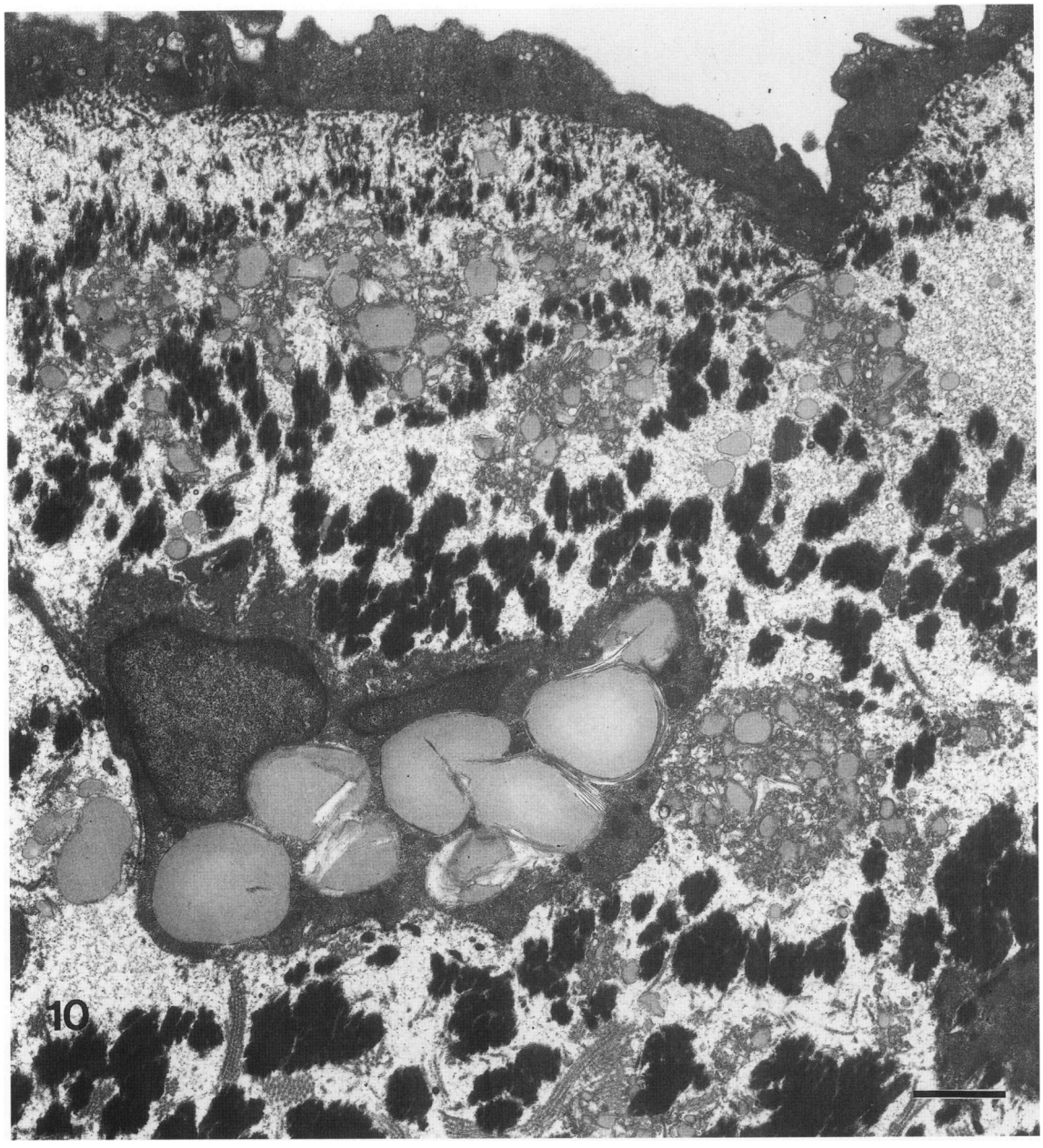


**Figure 10.** *Droplets of neutral lipid and membranous/granular material in the extracellular space near a smooth muscle derived foam cell. The broad distribution of the extracellular lipid, which extends to the bottom of the micrograph, is consistent with the hypothesis of direct extracellular lipid deposition. OTAP cytochemical processing at carotid branch point after 4 weeks of the diet. ×12,900, bar = 1 μm.*

tral lipid in the early lipid deposits, consistent with their derivation from cholesteryl ester-rich β-VLDL. Membranous lipid, however, appeared to be more abundant than neutral lipid in the deposits, confirming previous suggestions by Kruth[8] and by Simionescu et al.[9] Currently there is no explanation for the apparent abundance of membranous lipid—phospholipid and unesterified cholesterol—in the deposits, because either LDL or β-VLDL would be expected to contribute mostly neutral lipid in the form of cholesteryl ester. Studies of lipoprotein oxidation might provide an answer, but thus far the effects of oxidation on cholesteryl esters are not well defined.

## *Effects at Four Weeks*

After 4 weeks of atherogenic diet, both extracellular and cellular lipid deposition had progressed. Because of infiltration of the subendothelial space by monocyte-macrophages and subsequent uptake of lipid by these

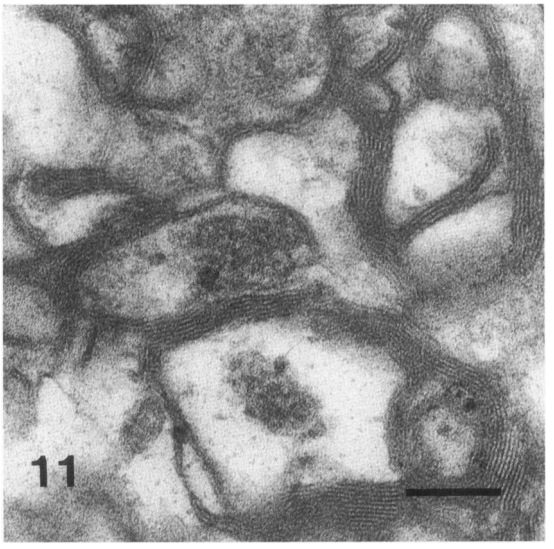


**Figure 11.** *Extracellular lipid deposition deep in the intima at 4 weeks of diet. OTAP cytochemistry demonstrates multilamellar vesicles which are ultrastructurally similar to those found in human atherosclerosis. Tissue site is the proximal aortic arch. ×132,000, bar = 100 nm.*

cells,[19] cellular lipid accumulation was much more extensive than extracellular. Nevertheless, the morphology of extracellular lipid deposits, comprising smaller droplets and vesicles rather than cellular deposits, and the location of the extracellular deposits, sometimes appearing interspersed with elastic fibers and sometimes appearing at the outer margins of foam cell lesions, suggested a continuing process of direct extracellular deposition even at this time.

## *Extracellular Lipid Deposition in Atherosclerosis*

The notion that tissue lipoproteins may directly contribute to extracellular lipid deposits, without an intervening process of lipoprotein uptake and lipid accumulation in cells, was first proposed by Smith and Slater in the late 1960s on the basis of detailed light microscopic and chemical analyses of human atherosclerotic lesions.[1] Among the evidence presented at that time was the fact that fatty acyl patterns of cholesteryl esters found in fibrous plaques tend to resemble the patterns found in plasma lipoproteins (rich in cholesteryl linoleate), rather than those of fatty streaks containing foam cell lipid (rich in cholesteryl oleate). Subsequent morphologic evidence favoring a major role for direct extracellular lipid deposition in human atherosclerosis has included the demonstration of lipid deposits interspersed with elastic fibers and with bundles of collagen fibrils,[2,3] the discrepancy between the superficial location of most foam cell infiltrates and the deep intimal location of extracellular lipid deposits in early aortic fibrous plaques,[26] and the small sizes of extracellular lipid droplets compared with cellular lipid droplets in early and mature fibrous plaques.[2,4] Interaction of lipoproteins with extracellular tissue elements—especially proteoglycans and elastin—has been demonstrated repeatedly.[27–32] Cholesterol-rich lipid vesicles, which by light and electron microscopy occur mostly in the extracellular space, have recently been isolated and characterized from human and rabbit atherosclerotic tissue.[33]

Fusion of lipoproteins in the extracellular space, forming larger lipid deposits, was proposed by Frank and Fogelman[11] on the basis of freeze-etch electron microscopy of atherosclerosis in cholesterol-fed rabbits and Watanabe Heritable Hyperlipidemic rabbits. Coalescence of lipoprotein lipid domains, forming neutral lipid droplets and membranous vesicles, also occurs when low-density lipoproteins are aggregated by vortexing.[17,34] It seems likely that lipoprotein aggregation and coalescence, induced by unknown mechanisms, accounts for the formation of both neutral and membranous extracellular lipid deposits in the rabbit model.

Studies on mechanisms and eventually prevention of extracellular lipid deposition in atherosclerosis will benefit greatly from the development and validation of an animal model. The current results add to evidence from earlier studies,[8–11,35] suggesting that the cholesterol-fed rabbit is such a model when appropriate observations are made during the period from 1 to 4 weeks after the onset of cholesterol feeding.

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