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ELECTRON MICROSCOPY OF LIPID DEPOSITS IN HUMAN ATHEROSCLEROSIS

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Abstract

The filipin probe associated with tannic acid stain was used to study intra- and extracellular lipids in surgically removed human atherosclerotic lesions (n = 20). In particular, intimal thickenings, fatty streaks and fibrolipidic plaques have been investigated by using mainly transmission and scanning electron microscopy. In the intimal thickenings, the lipid deposits were mainly localized in the subendothelial space as homogeneously sized particles (40-140 nm) and more heterogeneous uni- multilamellar vesicles (35-700 nm). Intermediate lipid forms were also observed. In the fatty streaks, the lipid deposits were intracellular and mainly observed in cells with a monocyte/macrophagic phenotype. Lipid inclusions, lipid lysosomal bodies and intracellular cholesterol crystals very similar to those observed in experimentally induced atherosclerosis were documented. In the fibrolipidic plaque the lipid deposits were found both in the intracellular and in the extracellular compartments. Lipids accumulated within arterial macrophages and smooth muscle cells, usually as lipid droplets. Clusters of lipoprotein-like particles (50 nm in diameter) as well as larger uni- multilamellar lipids (700 nm) with an occasional compound appearance were particularly observed bound to elastic tissue and collagen fibers. These morphological observations outline the complexity of lipid metabolism in the various histological aspects of human atherosclerosis.

KEY WORDS: Human atherosclerosis, Lipids, Tannic acid, Filipin, Scanning electron microscope, Transmission electron microscope.

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Introduction

The accumulation of lipids in the arterial intima represents one of the hallmarks of human atherosclerosis (6). Histologically, the fatty streaks consist of an intimal accumulation of cells with a foamy appearance, lipophages, contained between the endothelium and the inner elastic lamina, whereas the fibrolipidic plaques have a necrotic-lipidic core bounded by an extensive fibrous cap (1, 24). Clinically, hypercholesterolemia is the only established risk factor for the onset of atherosclerosis in man (20). Moreover, it has been suggested that the long standing lipid sequester may produce toxic effects on the cell and matrix components of the plaque thus resulting in its incipient rupture (25).

Despite this fact, there are very few satisfactory reports in literature on the ultrastructure of intracellular and even less of extracellular lipid deposits in human atherosclerosis (4, 7, 9, 10, 11, 21).

The ultrastructural appearance of lipids is dependent upon various factors, such as size, chemical composition of deposits and the method of tissue preparation (8). Lipids are particularly susceptible to the fixation and dehydration steps of routine preparation for electron microscopy. This may explain the difficulty of interpreting electron microscopic images of extracellular lipid deposits in conventionally processed specimens, which, at best, were described, in the early studies, as vesicles (21), droplets (4) and ring forms (9).

The application of reliable lipid cytochemical methods to the study of human atheromatous arterial lesions appears, therefore, of cardinal importance. Recently, some techniques have been introduced in order to facilitate the ultrastructural examination of such lipid deposits.

The presence of subendothelial

unesterified cholesterol-rich lipid vesicles, "liposomes", has been demonstrated in the early stages of experimentally induced atherosclerosis (23) as well as in atherosclerotic lesions of human aortas (7,15,16). These vesicles were detected by means of the probe filipin, which specifically binds to 3-β- hydroxycholesterol.

The use of the osmium-thiocarbohydrazide-osmium and tannic acid-paraphenylenediamine techniques (11) allowed to distinguish the extracellular lipid vesicles, with a high phospholipid and unesterified cholesterol content from the lipid droplets which are rich in cholesteryl esters in human fibrous plaques.

In this report, we used the filipin probe associated to tannic acid stain to study the lipid imbibition in human atheromatous arterial lesions at different stages of evolution, i.e., intimal thickenings, fatty streaks, fibrolipidic plaques. The pattern of the arterial lipid imbibition has been investigated by using transmission and scanning electron microscopy (TEM and SEM). We also used surgical material to minimize changes and alterations which can possibly be introduced in lipids by post-mortem phenomena.

Materials and Methods

Ten patients, 9 males and 1 female aged between 54 and 77 (average 63.8), with a previous history of cerebrovascular insufficiency underwent bilateral carotid thromboendarteriectomy (TEA). All the patients were smokers, 60% had arterial hypertension, 40% hyperlipidemia, 20% diabetes and ischemic cardiopathy.

Clinical examination and instrumental tests (Doppler echotomography and angiography) revealed the presence of a bilateral carotid lesion with a cerebral ischemic risk in all the patients (stenosis > 50% in 60% of the cases and morphological aspects of complicated plaque in 40%).

In all the patients surgical treatment consisted of a bilateral carotid TEA carried out in two steps (6 months interval between the two operations).

Samples were taken directly in the operating room from the grossly unaffected common carotid artery, at approximately 2 cm from the main lesion at the level of the carotid bifurcation.

The tissue fragments were cut into slices and then immersed in a pre-cooled (4° C) phosphate-buffered saline (PBS), containing 0.25% heparin. Subsequently, they were prefixed in 2% glutaraldehyde in cacodylate buffer 0.1 M, pH 7.4, for 10 min, incubated for 1 h at 22° C in the same fixative with the addition of 0.02 mg/ml of filipin (Sigma Chemical Company, St. Louis, Missouri), previously dissolved in dimethylformamide, and then fixed a second time in 2% glutaraldehyde in cacodylate buffer for 60 min.

The specimens for TEM observation were post-fixed in 1% osmium in cacodylate buffer 0.1 M for 90 min at 4° C, washed in the same buffer and finally treated with 1% tannic acid in cacodylate buffer 0.05 M, pH 7, for 30 min at 22° C. The specimens were then dehydrated in increasing concentrations of ethanol, transferred in propylene oxide and embedded in araldite. Semithin sections were taken from the embedded blocks for light microscopic investigation and ultrathin sections for ultrastructural examination. The thin sections, collected on the observation grids, were stained with uranyl acetate and lead citrate, covered with a thin layer of evaporated carbon and examined in a JEOL 100B TEM.

Fig.1. SE (1a) and reversed polarity BSE (1b) of an intimal thickening. The BSE image shows a granular subendothelial positivity in the areas of lipid deposition. SEM. Bar = 100 µm.

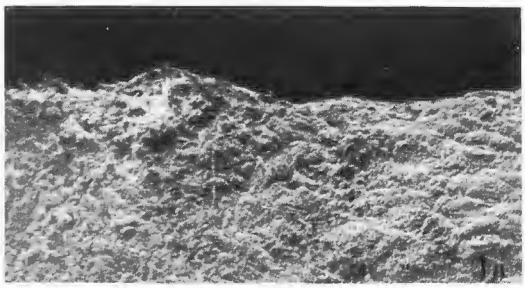
Fig.2. (+) BSE of the same specimen seen in Fig.1. At higher magnification individual lipid vesicles and droplets are visible. SEM. Bar = 10 µm.

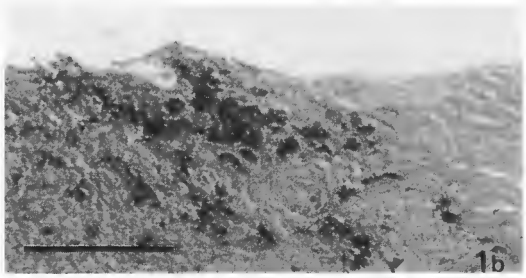
Fig.3. Intimal thickening. A large collection of homogeneously sized lipid particles (arrows) is observed next to the endothelial plasma membrane. The asterisk indicates a cytoplasmic vacuole containing particles. The arrowheads show foci of particles released. TEM. Bar = 300 nm.

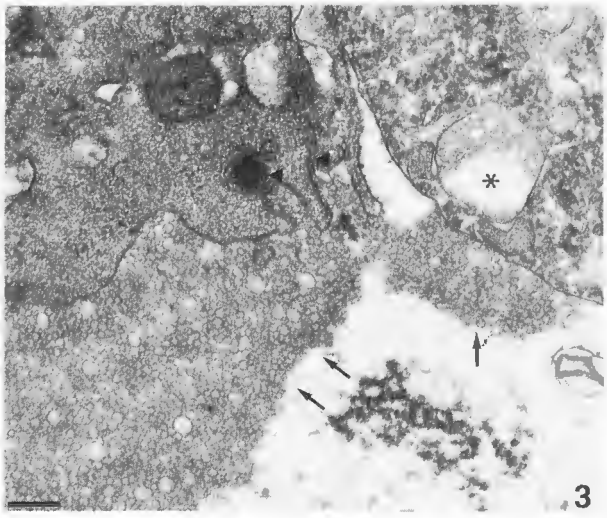
Fig.4. Intimal thickening. The asterisk marks numerous tightly packed particles featuring a clear center and a monolayer surface structure. The arrows indicate the concentrically arranged phospholipidic lamellae bounding a large extracellular lipid deposit. TEM. Bar = 200 nm.

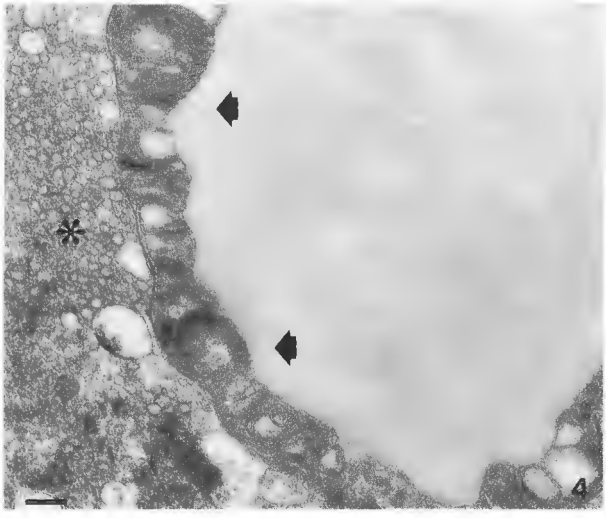
Fig.5. Intimal thickening. In the subendothelial space the particles (thin arrowhead) are enveloped by phospholipidic lamellae (arrow). A myelin figure is also seen (arrowheads). E: endothelium. TEM. Bar = 300 nm.

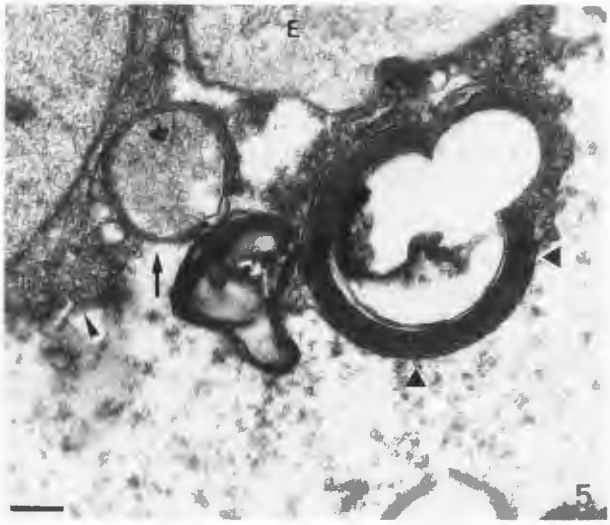
Fig.6. Intimal thickening. Loose aggregates of heterogeneous vesicles showing a clear or amorphous/granular center and uni- multilamellar periphery are shown. TEM. Bar = 500 nm.

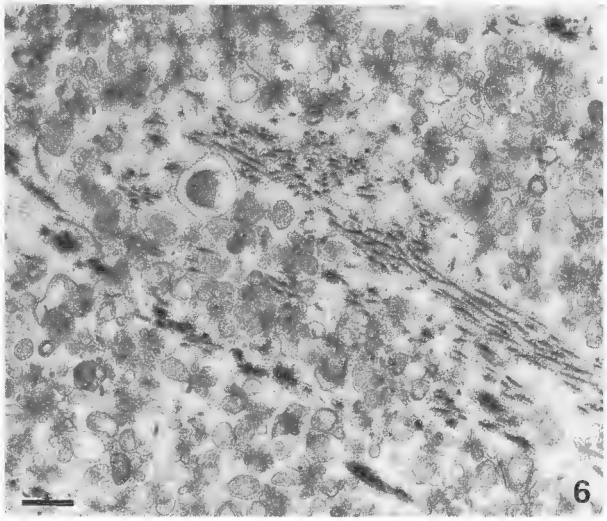


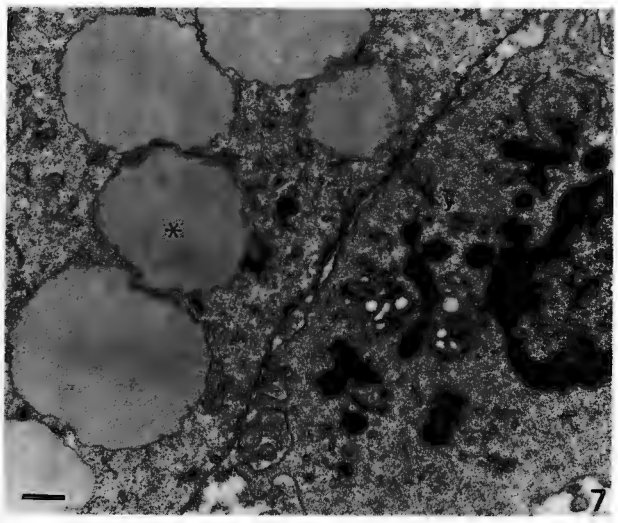


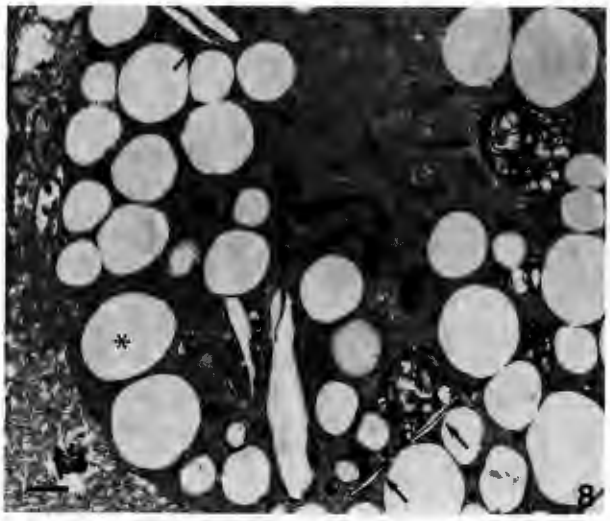












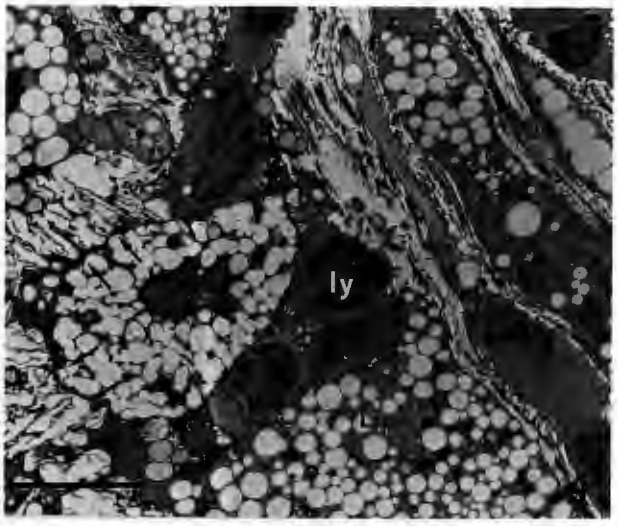


Fig.7. Fatty streak. A lipophage with numerous lipid inclusions (asterisk) is seen along with a large granular lymphocyte (Ly). The lipid periphery presents a crenated appearance. TEM. Bar = 10 µm.

Fig.8. Fatty streak. Different intracellular deposits are illustrated: lipid inclusions (asterisk), lysosomal lipid bodies (large arrow), cholesterol crystals (small arrow). TEM. Bar = 1 µm.

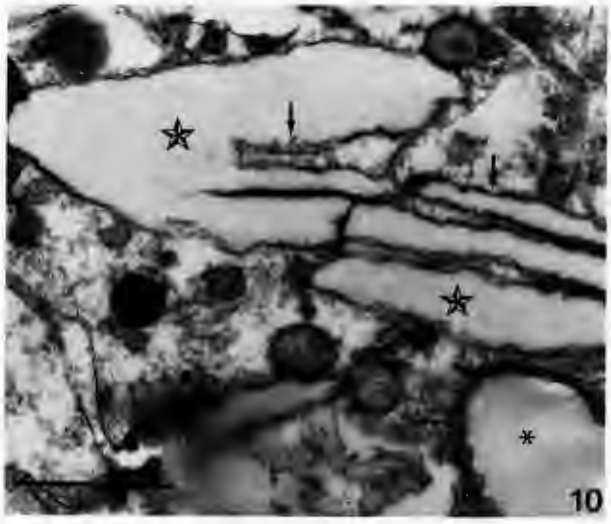


Fig.9. Fatty streak. At low magnification, the lesion is composed of lipophages (Lip) as well as satellite lymphocytes (Ly). The arrows indicate characteristic extracellular crenated cholesterol crystals most likely derived from cell necrosis. TEM. Bar = 10 µm.

Fig.10. Fatty streak. Single cholesterol crystals (stars) and lipid droplets (asterisk) are seen along with cell debris. The arrows show the crenated appearance of the delimiting lamellae. TEM. Bar = 1 µm.

The specimens for SEM observation were postfixed in osmium for 15 min. Incubation in tannic acid was followed by dehydration in acetone and by critical point drying. Subsequently, the specimens were mounted on carbon stubs, covered with a layer of evaporated carbon and finally observed with a Philips 505 SEM equipped with detectors for secondary (SE) and backscattered electrons (BSE).

The histological appearance of the carotid lesions examined revealed intimal thickening in 20% of the cases, a fatty streak in 15%, a fibrolipidic plaque in 40%, a fibrous plaque in 5% and a complicated lesion in 20%.

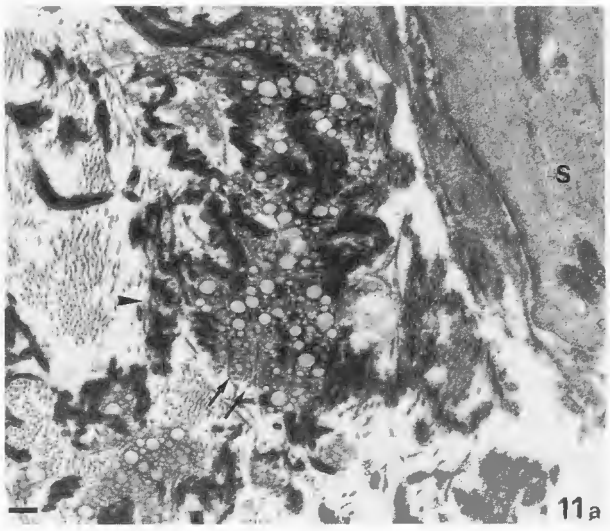
Results

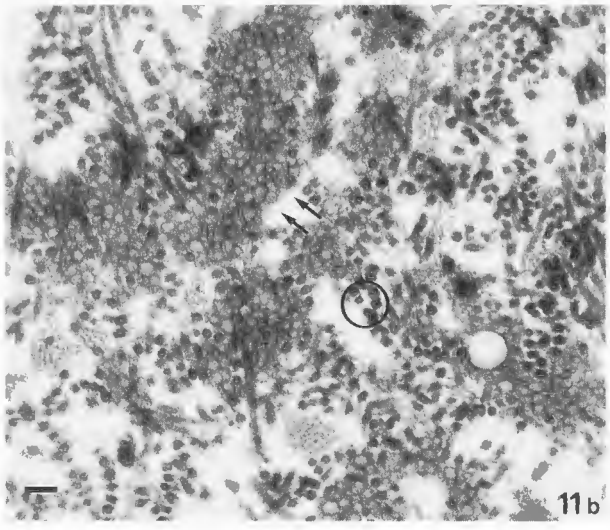
At SEM level, correlating SE and BSE images of the sectioned human arterial wall showed different patterns of lipid staining in relation to the histological appearance of the lesion examined. Intimal thickening associated to the main atherosclerotic lesion as well as fatty streaks showed a subendothelial granular staining (Fig. 1a,1b). At high magnification, individual lipid vesicles and droplets were recognized (Fig. 2). On the contrary, the raised plaques showed multiple foci of positivity beneath the fibrous cap. Most of these lesions revealed an unreactive necrotic core surrounded by a poorly stained zone. The TEM observation of adjacent areas showed that positivity was the result of the presence of extra- and intracellular lipid deposits.

In the intimal thickenings, the lipid deposits were mainly localized in the subendothelial extracellular space. TEM revealed clusters of tightly packed lipid particles in correspondence of the abluminal aspect of the endothelial cell membrane (Fig.3). The particles showed a homogeneous size (50-140 nm) and were surrounded by a monolayer structure presenting a slightly irregular outline (Fig. 4). Lipid particles were at times observed on the adluminal aspect of the endothelial cell membrane, in the interendothelial spaces as well as within vacuoles and cystic lacunae of endothelial cells and intimal macrophages. In the subendothelial space, the particles appeared occasionally sequestered in large structures which were bounded by numerous tightly stacked lamellae (Fig. 5).

In addition, loose aggregates of more heterogeneous lipid vesicles (35-700 nm in diameter), with a clear or granular center and uni- multilamellar periphery (Fig. 6) were also observed.

In the fatty streaks, the lipid deposits were for the most part intracellular and were mainly observed in elements with a monocyte/macrophagic





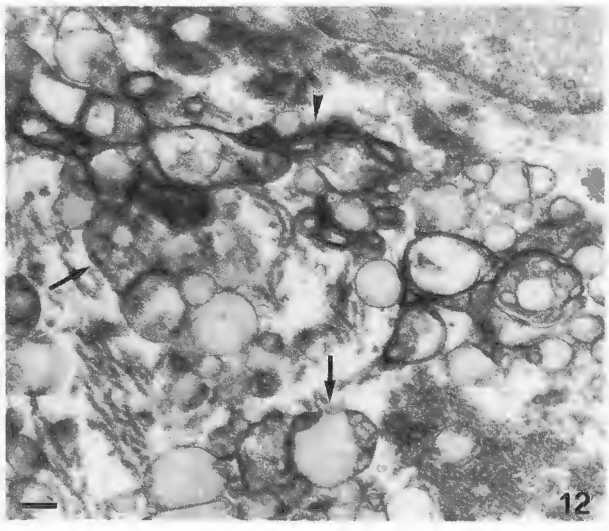


Fig.11. Fibrolipidic plaque. Below the fibrous cap clusters of lipid particles (arrows) are associated to elastic fibrils (arrowhead) (Fig. 11a) and to collagen fibers (circle) (Fig. 11b). In Fig. 11a "S" indicates a smooth muscle cell. TEM. Bar: 11a = 300 nm; 11b = 150 nm.

Fig.12. Fibrolipidic plaque. A collection of large lipid vesicles presenting a lamellar periphery (arrowhead) and a pitted or compound appearance (arrows) is seen. TEM. Bar = 200 nm.

phenotype. The lipid inclusions were the most commonly found intracellular deposits. These inclusions, moderately electron dense, were not bounded by a true trilaminar membrane, but an osmiophilic membrane-like structure, with an occasional crenated appearance, was seen around the lipid droplets (Fig. 7). The lysosomal lipid bodies, true secondary lysosomes, have a single peripheral membrane. They consist of laminated membranous structures showing a stacked, or whorled arrangement (Fig. 8). They may contain minute cholesterol crystals. The intracellular cholesterol crystals (Fig. 8) appear contained in the remains of lysosomal lipid bodies. They appear in large intimal lipophages and are frequently associated with features of cell degeneration.

On the contrary, the extracellular lipid deposits were rarely found and frequently assumed a crystalline appearance. This aspect was particularly obvious around the large cellular clusters consisting of lymphocytes and of degenerating lipophages, with pyknotic nuclei and cytoplasmic autophagic vacuoles (Fig. 9). The crystals were seen in conjunction with cell debris, myelinoid membranes and osmiophilic granular material. Moreover, they showed a bizarre morphology like the tip of an arrow or a trident and were bounded by a crenated membranous lamella (Fig. 10).

In the fibrolipidic plaques the lipids were detected both in the extracellular and in the intracellular compartments. The extracellular lipid deposits were mainly observed in correspondence of extracellular matrix components below the fibrous zone. Clusters of electron lucent particles (Figs. 11a, 11b) were seen, with a homogeneous diameter (average ⌀ 50 nm) along with large uni- multilamellar lipids (average ⌀ 700 nm) (Fig. 12). Pitting of the large lipid deposits was also evident. The elastic tissue showed a marked affinity for these lipid deposits. The split inner elastic lamina displayed a particular strained appearance due to the presence of minute lipid droplets presenting occasional calcific periphery. There was also evidence of perilipidic calcification in the surrounding lipidic aggregates.

The intracellular lipids were observed as lipid droplets in foam cells of both macrophage and smooth muscle cell origin.

Discussion

This study concentrates upon lipid ultrastructure in different stages of human atherosclerosis. According to Simionescu et al. (23) we used the filipin probe followed by tannic acid impregnation in order to improve the electron microscopic appearance of atherosclerotic lipid deposits. Tannic acid acts as a mordant and, therefore, improves the staining intensity of lipid membranes upon subsequent application of heavy metal salts, e.g., uranyl and lead salts (22). As a consequence of this, in our study the presence of uni-multilamellar lipid deposits was clearly revealed. Filipin, a polyene antibiotic, allows the identification of the structures in which 3-β-hydroxysterols are localized in an accessible position (5). The presence of specific filipin-cholesterol complexes produces characteristic membrane protusions which are visible at TEM level as ondulating profiles. However, in our series, the filipin-sterol complexes were occasionally observed. Our TEM images revealed a crenated lipid outline only in some intracellular lipid inclusions (see, Fig. 7) and in extracellular cholesterol crystals (see, Fig. 10). The failure of lipid deposits to react with filipin may be related to different causes. TEM is not the ideal microscopic imaging mode to reveal these complexes. Artifacts induced by filipin are better appreciated in freeze-fracture replicas as protuberances or membrane depressions and in negatively stained preparations as holes or pits (7). Moreover, filipin has a limited penetration into the tissue block and easily decomposes (2). Finally, the potential formation of markedly crenated lipid deposits, such as those illustrated in Fig. 7, by initial glutaraldehyde fixation cannot yet be ruled out. These limitations will require further investigation.

In this report we have described different ultrastructural aspects of lipid accumulation in human atherosclerosis. At our personal knowledge, this is the first paper describing the lipid ultrastructure in relation to all the significant histological aspects of the human arterial disease.

Basically, the ultrastructural observation of the intimal thickenings located on the border with the main plaques revealed mainly extensive extracellular fatty deposits. Some of the subendothelial deposits consisted of minute clusters of tighly packed lipid particles localized in correspondence of the abluminal aspect of the endothelial cell membrane. These particles were homogeneous in size (50-140 nm). They were characterized by a translucent core and by a slightly irregular monolayer surface structure. The particles were also observed on the luminal aspect of endothelial membrane, in the intercellular spaces, as well as within cytoplasmic vacuoles of endothelial cells and subendothelial macrophages. The

precise nature of these vesicles is unknown. However, Hoff and Gaubatz (12) have shown the presence of LDL-like lipoproteins in homogenates of both grossly normal intima and fibrolipidic plaques from human aortas. It may be therefore speculated that vesicles of this nature are derived from chemically modified plasma low and/or very low density lipoproteins.

Another finding of the intimal thickenings was the presence of loose aggregates of uni- multilamellar vesicular structures (35-700 nm in diameter) showing a clear or amorphous/granular center. The morphology of these vesicles is very similar to that of the vesicles Simionescu et al. (23) termed "extracellular liposomes". At the ultrastructural level, these authors were able to recognize, as a prelesional stage of experimentally induced atherosclerosis, the presence of peculiar subendothelial lipid vesicles (liposomes) having diameters ranging from 100-300 nm, electron transparent cores and uni-multilamellar peripheries. Combined cytochemical and immunocytochemical methods demonstrated that liposomes are particularly rich in unesterified cholesterol, apoprotein-B and phospholipids (19).

Unesterified cholesterol-rich lipid particles have also been described by Kruth in early (16) and advanced (15) human aortic atherosclerotic lesions using fluorescent microscopy. In a subsequent study (7), Chao et al. were able to isolate the unesterified cholesterol-rich lipid particles from human and cholesterol-fed rabbit aortas. The isolated particles, having a high molar ratio of unesterified cholesterol to phospholipid and a high percentage of their cholesterol in an unesterified form, had diameters between 70-300 nm and showed unilamellar and multilamellar periphery. After incubation with filipin, most of the particles showed typical filipin-sterol complexes.

Concerning various similarities, e.g., diameters, electron miscroscopic appearance, subendothelial localization, we suspect that the lipid vesicles observed in our series may correspond to the liposomes described by Simionescu et al. (23) and by Chao et al. (7).

In addition, large multilamellar structures containing 50-140 nm particles were at times seen in the subendothelial space. These compound lipids may represent a transitional structure between the lipid particles and the liposome-like structures previously described. This finding raises yet another question, whether the assembly of the uni- multilamellar vesicles occurs within the extracellular space or within the cells.

In contrast, fatty streaks are mainly characterized by an extracellular pattern of lipid deposition. Our electron microscopic results agree with those provided by Lupu et al. (18) who studied the onset of fatty lesions in cholesterol-fed rabbits using physical, cytochemical and ultrastructural methods. Lipid inclusions, lipid lysosomal bodies and intracellular cholesterol crystals have been clearly observed in human fatty streaks. Non-membrane bound lipid droplets particularly filled the cytoplasm of monocyte/macrophagic cells and occasionally that of smooth muscle cells. The transition to tissue macrophages was associated with the cytoplasmic appearance of lipid lysosomal bodies containing concentrically arranged multilamellar structures. The presence of phospholipidic coatings has been considered as a barrier limiting the turnover of the crystalline cholesterol, thus rendering the fatty lesions less susceptible to regression (18). Lewis et al. (17) described an increase in the macrophage lysosome number and size concomitant with lesion progression in the hypercholesterolemic White Carneau pigeons. In florid fatty lesions, we observed the presence of large intimal lipophages containing intracellular cholesterol crystals within remains of lipid lysosomal bodies. The crystals probably originate from the precipitation of the surplus cytoplasmic cholesterol following the functional breakdown of the lysosomes. The progressive involvement of lysosomes may be, therefore, responsible for the progressive macrophage failure in removing the fatty excess from the arterial wall. The cells may undergo necrosis, also due to satellite lymphocytic aggression, and release fatty substances which contribute to the pool of extracellular lipids. The fatty release may also account for the presence of numerous crenated crystals which we observed in correspondence of the degenerating lipophages.

In the fibrolipidic plaque the lipid deposits may be found in a number of areas (24) as well as in various physical states (14). Lipid accumulates within arterial macrophages and smooth muscle cells, usually as lipid droplets. However, a substantial amount of lipid may also be found in the extracellular space. In our series, the extracellular lipid deposits were mainly observed bound to elastic tissue and collagen fibres. This finding further supports the view that the interaction between lipids and extracellular matrix components, namely elastin, collagen and proteoglycans play an important role in extracellular lipid deposition in human arteries (3,10,13). Clusters of electron lucent particles (average diameter 50 nm) were observed

in conjunction with larger uni-multilamellar lipids (average diameter 700 nm) frequently showing a pitted or compound appearance. Pitting or blebbing of extracellular fatty deposits have been disclosed by Guyton and Klemp (11) in human fibrous plaques using complementary lipid cytochemical methods. The presence of extracellular hybrid lipid structures featuring an oily and membranous phase has been, therefore, postulated and considered an expression of ongoing extracellular metabolism of lipid in the lesion.

These observations have shown the ultrastructural appearance of intra- and extracellular lipid deposits in the various histological aspects of human atherosclerosis. Lipid interactions with cells and extracellular matrix components of the arterial wall have been documented. Some morphological features suggesting a direct growth of lipid in the extracellular space have been also presented. The sequence and relative contribution of each of these phases to the development of stable and irreversible changes in the chemical and physical state of the parietal lipid pool remain to be established.

References

1. Adams CWM (1987). Disordered structure and function in the atherosclerotic artery. In: Atherosclerosis-Biology and Clinical Science. AG Olsson (ed.). Churchill Livingstone, Edinburgh, pp. 75- 88.

2. Behnke O, Tranum-Jensen J, van Deurs B (1984). Filipin as a cholesterol probe: I.Morphology of filipin-cholesterol interaction in lipid model systems. Eur J Cell Biol, 35:189-199.

3.Bihari-Varga M (1987). Proteoglycans, glycosaminoglycans and lipid deposition. In: Atherosclerosis - Biology and Clinical Science. AG Olsson (ed.). Churchill Livingstone, Edinburgh, pp.145-146.

4. Bocan TMA, Schifani TA, Guyton JR (1986). Ultrastructure of the human aortic fibrolipidic lesion: formation of the atherosclerotic lipid-rich core. Am J Pathol, 123:413-422.

5. Börning H, Geyer G (1974). Staining of cholesterol with the fluorescent antibiotic "filipin". Acta Histochem, 50: 110-116.

6. Buck RC, Rossiter RJ (1951). Lipids of normal and atherosclerotic aortas. Arch Pathol, 51:224-237.

7. Chao FF, Amende LM, Blanchette-Mackey EJ, Skarlatos SI, Gamble W, Resau JH, Mergner WT, Kruth HS (1988). Unesterified cholesterol-rich lipid particles in atherosclerotic lesions of human and rabbit aortas. Am J Pathol, 131:73-83.

8. Ghadially FN (1988). Ultrastructural pathology of the cell and matrix. IIIrd ed., Butterworths, London, pp.974-977.

9. Ghidoni JJ, O'Neal RM (1967). Ultrastructure of the human atheroma. Exp Mol Pathol, 7:378-400.

10. Guyton JR, Bocan TMA, Schifani TA (1985). Quantitative ultrastructural analysis of perifibrous lipid and its association with elastin in non-atherosclerotic human aorta. Arteriosclerosis, 5:644-652.

11. Guyton JR, Klemp KF (1988). Ultrastructural discrimination of lipid droplets and vesicles in atherosclerosis: value of osmium-thiocarbohydrazide-osmium and tannic acid-paraphenylenediamine techniques. J Histochem Cytochem, 36:1319- 1328.

12. Hoff HF, Gaubatz JW (1982). Isolation, purification, and characterization of a lipoprotein containing apo B from the human aorta. Atherosclerosis, 42:273-297.

13. Hornebeck W, Robert L (1987). Elastin-lipid interaction in atherogenesis. In: Atherosclerosis - Biology and Clinical Science. AG Olsson (ed.). Churchill Livingstone, Edinburgh, pp.147-149.

14. Katz SS, Small DM (1980). Isolation and partial characterization of lipid phases of human atherosclerotic plaques. J Biol Chem, 255:9753-9758.

15. Kruth HS (1984). Histochemical detection of unesterified cholesterol within human atherosclerotic lesions using the fluorescent probe filipin. Atherosclerosis, 51:281-292.

16. Kruth HS (1985). Subendothelial accumulation of unesterified cholesterol. An early event in atherosclerotic lesion development. Atherosclerosis, 57:337-341.

17. Lewis JC, Taylor RG, Ohta K (1988). Lysosomal alterations during coronary atherosclerosis in the pigeon: correlative cytochemical and three-dimensional HVEM/IVEM observations. Exp Mol Pathol, 48 (1):103-115.

18. Lupu F, Danaricu I, Simionescu N (1987). Development of intracellular lipid deposits in the lipid-laden cells of atherosclerotic lesions. A cytochemical and ultrastructural study. Atherosclerosis, 67:127-142.

19. Mora R, Lupu F, Simionescu N (1987). Prelesional events in atherogenesis. Co-localization of apoprotein B, unesterified cholesterol and extracellular phospholipid liposomes in the aorta of hyperlipidemic rabbit. Atherosclerosis, 67: 143-154.

20. Pollack OJ (1987). Risk factors for atherosclerosis in proper perspective. Atherosclerosis, 63:257-262.

21. Ross R, Wight TN, Strandness E, Thiele B (1984). Human atherosclerosis. I. Cell constitution and characteristics

of advanced lesions of the superficial femoral artery. Am J Pathol, 114:79-93.

22. Simionescu N, Simionescu M (1976). Galloylglucoses of low molecular weight as mordant in electron microscopy. I. Procedure and evidence for mordanting effect. J Cell Biol, 70:608-621.

23. Simionescu N, Vasile E, Lupu F, Popescu G, Simionescu M (1986).Prelesional events in atherogenesis. Accumulation of extracellular cholesterol-rich liposomes in the arterial intima and cardiac valves of the hyperlipidemic rabbit. Am J Pathol, 123:109-125.

24. Smith EB, Evans PH, Downham MD (1967). Lipid in the aortic intima. The correlation of morphological and chemical characteristics. J Atheroscl Res, 7:171-186.

25. Tracy RE, Kissling GE, Malcom GT, Devaney K (1987). Sequestration hypothesis of atherosclerosis. Virchow Arch A, 411:425-434.

Discussion with Reviewers

W.G. Jerome: You state that cholesterol crystals were particularly evident around degenerating lipophages and lymphocytes. What criteria did you use to determine degenerating cells?
Authors: We used the following morphological criteria: i) pyknosis of nuclei, ii) swelling and/or piknosis of mitochondria, iii) presence of autolysosomes containing remains of cytoplasmic structures, iv) cytoplasmic shedding, v) loss of membrane integrity.

W.G. Jerome:The large lipid accumulations you show in figure 8 are of a similar size to the large lipid filled lysosomes we have reported in foam cells from pigeon atherosclerotic lesions (Jerome & Lewis, Am J Pathol, 119:210, 1985; Jerome & Lewis, Am J Pathol, 128:253, 1987). We have also shown that in pigeons the size of free cytoplasmic inclusions remains relatively constant at 1.3 micrometers or less. Since lipid can often disquise the presence of membranes unless special staining procedures are employed, I am curious to know if you think some of the large lipid accumulations in your foam cells might not be early secondary lysosomes.
Authors: It is conceivable that part of the intracellular lipid inclusions are early secondary lysosomes. However, we did not perform a specific serial study on the same tissue block. Therefore, we cannot substantiate your observation.

J. Berliner and J Frank: In the definitive fatty streak, is there a difference in appearance of extracellular lipids near the endothelium as opposed to deep in the lesion?
Authors: In the fatty lesions, the lipid deposits appeared to be for the most part intracellularly located. Thus, we had no sufficient data to detect significant differences between the subendothelial and the more internal extracellular lipids.

J. Berliner and J Frank: Is there any evidence from particle size that lipophages take up by phagocytosis large numbers of lipid particles?
Authors: Yes, there is. We have some micrographs showing macrophages while taking up clustered lipid particles by means of surface flaps. In the same cells, phagosomes containing particles are present in the cytoplasm.

N. Simionescu: How well preserved is the endothelium in your material?
Authors: In the present study the specimens were prefixed by submersion in the operating room. As specifically discussed in a previous paper (Pasquinelli et al., Endothelial injury in human atherosclerosis, Scanning Microsc. 1989, 3(3), 971-982), this procedure gives a sufficiently good endothelial preservation. A better morphology can be clearly achieved by using a perfusion fixation protocol. However, legal and ethical considerations prevented us from using this approach.