Development of the Atherosclerotic Core Region

Chemical and Ultrastructural Analysis of Microdissected Atherosclerotic Lesions From Human Aorta

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Abstract  Lipid deposits in human atherosclerotic fibrous plaques exhibit marked differences in chemistry and ultrastructure from lipid deposits in fatty streaks, leading some investigators to question whether fibrous plaques originate from fatty streaks. To examine lesion transition, we employed lipid microanalysis, electron microscopy, and immunohistochemistry on fatty streaks, fibrolipid lesions (small raised lesions), and fibrous plaques from human aorta. Both fatty streaks and caps of fibrolipid lesions were high in esterified cholesterol content (mean, 62% of total cholesterol) and high in cholesteryl oleate content compared with cholesteryl linoleate content. Fatty streaks and fibrolipid lesion caps also showed similar morphology, characterized mostly by macrophage-derived foam cells in the superficial intima. Core lipids in both small and large raised lesions differed markedly from this pattern. Fibrolipid lesion cores showed mostly vesicular extracellular deposits, sometimes accompanied by cholesterol clefts, while fibrous plaque core deposits were also extracellular but had a variable appearance. Compared with fatty streaks, fibrolipid lesion cores showed significantly increased free/total cholesterol fractions (66%) and decreased fractional contents of cholesteryl oleate. Fibrous plaque cores had variable distributions of free and esterified cholesterol but significantly decreased cholesteryl oleate fractions compared with fatty streaks. The results support the concept of lesion transition, which is marked by deep intimal, extracellular deposition of cholesterol-rich, vesicular lipid deposits in small raised lesions. In the core region of larger raised lesions, both cholesterol-rich and cholesteryl ester–rich lipid deposits appear to form in the extracellular space.

Key Words • atherosclerosis • fatty streak • fibrous plaque • cholesterol • cholesteryl esters

Lipid deposition in human arterial fatty streaks consists mostly of cholesteryl esters, of which cholesteryl oleate is the most prominent, occurring primarily in cytoplasmic lipid droplets with droplet profile diameters generally exceeding 0.4 μm. Lipid deposits in large, mature fibrous plaques, in contrast, are often enriched in free cholesterol, have cholesteryl linoleate as the most prominent cholesteryl ester, and are found predominantly in the extracellular space in the form of small lipid droplets (less than 0.4 μm profile diameter) and vesicles. These discrepant features have provided the impetus to question (1) whether the fibrous plaque core originates from lipids accumulated in foam cells via death of the cells and (2) whether fibrous plaque lesions have their origin in preexisting fatty streaks.

Human fibrous plaque core lipids may primarily originate from direct deposition of lipoprotein lipids in the extracellular space rather than from dead foam cells. This suggestion is based on observations of the site of lipid deposits in the smallest detectable core regions, the small sizes of lipid particles in core regions, cholesteryl ester fatty acyl patterns, binding of lipoproteins to the extracellular matrix, and the demonstration of lipoprotein fusion. However, no previous studies, including our own, have examined in detail the chemistry of lipid deposits in the early core region of fibrolipid lesions (small raised lesions) in human aorta. Such lesions, although they are simply small fibrous plaques by definition, may represent an important transitional stage in atherosclerosis development.

By the use of special lipid-preserving cytochemical techniques in electron microscopy, two patterns of lipid deposits have been identified in the core region of mature human aortic fibrous plaques: a vesicle-rich pattern, which is interspersed with cholesterol crystals and is presumed to have a high content of free cholesterol, and a droplet-rich pattern, which is presumed to have a high content of cholesteryl ester. Chemical lipid measurements are needed to confirm the distinction between the two patterns. A key question concerns which of these lipid deposition patterns may occur first in the development of the core region.

To address the questions raised above, we performed a study that combined chemical and ultrastructural analysis of lipids in human aortic fatty streaks, fibrolipid lesions, and fibrous plaques. The latter two lesion types were microdissected into cap and core regions prior to chemical analysis by capillary column gas chromatography. New techniques for electron microscopy of lipid deposits were used to determine, for the first time, the ultrastructural pattern of early core lipid deposits and to provide structural correlations with lipid analysis. Representative lesion slices were also embedded in paraffin and subjected to immunostaining for muscle- and macrophage-specific antigens to gauge the participation of these cell types in the formation of the early core region.
The results suggest that the superficial intima or cap of the fibrolipid lesion is similar to the fatty streak in terms of chemistry, the location of lipid in cytoplasmic droplets, and a high participation of macrophages in foam cell formation. The deep intima of fibrolipid lesions, where early core regions are found, consistently had a high content of free cholesterol and often had a cholesteryl ester fatty acyl pattern similar to the fibrous plaque core region. The major cell type in this area varied. Combining electron microscopic and chemical observations, the early core showed abundant extracellular lipid with a vesicular pattern rich in unesterified cholesterol, while paradoxically the more mature core region of fibrous plaques was quite variable, sometimes showing mostly vesicular lipid and sometimes a cholesteryl ester-rich droplet pattern of lipid deposits. Overall, these observations supported the hypothesis of an extracellular origin for core lipid deposits and were also compatible with the hypothesis of conversion from fatty streak to fibrous plaque in atherosclerotic lesion development.

Methods

Selection of Specimens

Human aortas were obtained at autopsy from the Harris County Medical Examiner in Houston, Tex, or the North Carolina State Medical Examiner in Chapel Hill, NC. Eighteen aortas were obtained from 14 male and 4 female subjects who ranged in age from 16 through 58 years (average, 35.6 years). The cause of death was generally trauma. Specimens were immersed in phosphate-buffered saline, pH 7.4, for 30 minutes. This was followed by a 5-minute rinse in 0.05 mol/L cacodylate-buffered 1% sodium sulfate, pH 7.4, containing 20 mmol/L butylated hydroxytoluene (BHT) to minimize autoxidation. Aortas were opened longitudinally, and three rapid changes of 70% ethyl alcohol, and then treatment with 1% paraphenylenediamine in 70% ethyl alcohol for 30 minutes. The free sterols from this reaction were separated after being vortexed in a hexane-water mixture, dried under nitrogen, reconstituted in hexane, and injected directly in the gas chromatograph. To quantify cholesteryl ester, an additional 0.5 mL of the steryl ester solution was saponified in 500 µL 0.5 mol/L methanolic KOH at 80°C for 30 minutes. The free sterols from this reaction were separated after being vortexed in a hexane-water mixture, dried under nitrogen, reconstituted in hexane, and chromatographed. Quantification was by comparison of peak areas of cholesterol and stigmastere, the latter resulting from saponification of internal-standard stigmasteryl ester. To determine cholesteryl ester fatty acids, transmethylation was performed on dried steryl ester eluates by adding 200 µL each of methanol, methanolic base (Supelco, Inc), and boron trifluoride (12% wt/wt in methanol; Supelco) and heating at 110°C for 30 minutes.

Chromatography was performed on a Hewlett-Packard 5890 gas chromatograph by using a 30-m Alltech capillary column coated with polyethylene glycol (Carbowax). Sterols were quantified isothermically at 260°C. Fatty acid methyl esters were quantified under the following chromatographic conditions: 150°C for 8 minutes, 3°C/min to 190°C, and 190°C for 15 minutes. The use of capillary column gas chromatography allowed chemical analyses to be performed on tissue samples weighing as little as 3 mg, small enough to define the early core region.

Immunohistochemical Identification of Cell Types

One-millimeter slices of aortic tissue from various lesions were fixed in methyl Carnoy's solution and embedded in paraffin. Adjoining sections were cut for staining with HHF35 human macrophage monoclonal antibody (both from Enzo Diagnostics) by a streptavidin-biotin peroxidase technique. Substitution of nonimmune mouse serum for the primary antibody resulted in essentially no visible reaction product on the sections.

Electron Microscopy

After slicing the tissue, representative lesion slices were bisected and subsequently processed by both the osmium-tannic acid–paraphenylenediamine (OTAP) and osmium-thio-carbohydrate-oxide (OTO) techniques for electron microscopy. Briefly, tissue blocks were fixed overnight in 0.1 mol/L cacodylate buffer, pH 7.4, containing 3% glutaraldehyde at 4°C. All subsequent steps were at room temperature. For the OTAP technique, tissues were postfixed in cacodylate-buffered 1% osmium tetroxide for 2 hours followed by rinsing and mordanting with 1% tannic acid in 0.05 mol/L cacodylate buffer, pH 7.4, for 30 minutes. This was followed by a 5-minute rinse in 0.05 mol/L cacodylate-buffered 1% sodium sulfate, three rapid changes of 70% ethyl alcohol, and then treatment with 1% paraphenylenediamine in 70% ethyl alcohol for 30 minutes. The blocks were dehydrated rapidly in graded ethyl alcohol and embedded in LX112 epoxy resin (Ladd Industries). For the OTO technique, tissues were postfixed in 2% cacodylate-buffered osmium tetroxide for 30 minutes, rinsed thoroughly, then treated for 5 minutes with freshly prepared, filtered 1.5% aqueous thio-carbohydrate-oxide (Sigma). This was followed by thorough rinsing and reimmersion in 2% buffered osmium tetroxide for 30 minutes. Dehydration and embedding were similar to the use steps in the OTAP method.
Semithin epoxy sections of 1 to 3 mm were cut with glass or diamond knives and stained with toluidine blue–basic fuchsin. Thin sections were obtained by using an LKB Ultratome III ultramicrotome and a Diatome diamond knife. Thin sections were stained with 7% uranyl acetate in 50% ethanol and with Sato’s lead citrate and were viewed on a JEOL 200CX or JEOL 1200EX electron microscope operating at 80 keV or 60 keV, respectively.

**Statistical Analysis**

The key question that this study asked was whether lipids in fatty streaks differ from lipids in the core regions of raised lesions. Therefore, the quantitative data describing fractions of free/total cholesterol and fractions representing cholesteryl linoleate (18:1/[18:1 + 18:2]) were tested by ANOVA involving fatty streaks, fibrolipid core regions, and fibrous plaque core regions using the Bonferroni adjustment for multiple comparisons (Sigmastat Statistical Program, Jandel Scientific).

**Results**

**Light Microscopy**

After OTAP processing and the preparation of epoxy-embedded sections, cellular lipid had the form of dark brown to black dense deposits, while extracellular lipid had a variable appearance of diffuse light brown staining, small dark punctate deposits, or less commonly, large dense deposits. Crystalline cholesterol was not preserved by either OTAP or OTO processing, but typical clear crystal-shaped clefts were found in some lesions. Almost all 13 fatty streaks examined by light microscopy (from 13 aortas) contained predominantly cellular lipid deposits, usually located near the luminal surface. Two fatty streaks contained small, deep intimal areas with extracellular lipid, and one contained cholesterol clefts in a small collagenous area in the intima abutting the internal elastic lamina. One additional lesion, obtained from an area of extensive and contiguous fatty streaking, had a thickened intima with abundant cellular and extracellular lipid, including a well-developed deep, hypocellular core region with many cholesterol clefts. These advanced features, found in lesions with the flat, surface appearance of fatty streaks, occurred in approximately the same proportion as in a previous study that dealt exclusively with grossly identified fatty streaks.

We have previously characterized fibrolipid lesions as small, raised, lipid-containing lesions that almost always contain both superficial foam cells and a deep intimal core region. Among the 14 lesions examined in the present study (from 10 aortas), one had no apparent extracellular lipid and therefore no lipid-rich core. Seven lesions had cholesterol clefts in the deep intima, and the remaining six lesions had light brown extracellular staining in the deep intima, indicative of a lipid-rich core (confirmed by electron microscopy). The core was usually hypocellular and occasionally acellular. With one or two exceptions, core lipids appeared to be mostly extracellular.

Unlike their constant appearance in the cap of fibrolipid lesions, foam cells were variably present in the cap of fibrous plaques. The 11 fibrous plaques examined by microscopy (from seven aortas) all contained core regions, but the appearance of the core varied from light brown to dark brown lipid staining. Most plaques showed cholesterol clefts in the core.

**Identification of Cell Types**

A total of 11 lesions were examined by immunostaining with HHF35 and HAM56 antibodies to identify smooth muscle cells and macrophages, respectively. Among these three fatty streaks and two fibrolipid lesions with small cores, macrophages were largely confined to the more superficial intimal sublayer, which has been termed the proteoglycan-rich layer. A few macrophages had penetrated the deeper, musculoelastic layer of the intima. Smooth muscle cells were present to a variable extent in the proteoglycan-rich layer, but they were always densely arranged in the musculoelastic intimal layer and in the tunica media (Figs 1 and 2). In the lesions with larger cores (two fibrolipid lesions and four fibrous plaques), macrophages were found throughout the core and at any depth in the intima. Determining the exact relation of macrophages and smooth muscle cells to core lipid deposits was difficult because the latter are removed by the delipidating solvents used in paraffin embedding. The OTAP procedure to visualize lipid was not employed with these tissue slices, since antigens are poorly preserved after osmium fixation.

**Electron Microscopy**

In lesions with small core regions, the general location of cellular lipids in the superficial intima and extracellular...
lar lipids in the deep intima was confirmed by electron microscopy. A striking demarcation was occasionally noted at the inner limiting elastic membrane, which divides the superficial, proteoglycan-rich layer from the deep, musculoelastic layer of the intima (Fig 3).

Cellular lipid deposits consisted of both membrane-bounded complex lipid structures, which were assumed to be lysosomes, and homogeneous cytoplasmic neutral lipid (cholesteryl ester) droplets. In the superficial intima, macrophages and macrophage-derived foam cells could be recognized by micropodia and plasmalemmal invaginations as well as the lack of a surrounding basement membrane. However, most foam cells were of indeterminate origin by electron microscopic criteria. In deep intima, large lipid droplets appeared in definite smooth muscle cells containing myofilaments, peripheral dense attachment sites, and surrounding basement membrane.

Extracellular lipid deposits showed all the various appearances previously described. The small core regions found in fibrolipid lesions, which were light brown by light microscopy, contained mostly vesicular lipid, best demonstrated in OTAP-processed specimens (Figs 4 and 5). Collagen bundles were often strikingly infiltrated with combined vesicular and droplet lipid. Cholesterol clefts appeared most often in combination with vesicles (Fig 6), but sometimes in juxtaposition to well-preserved neutral lipid and sometimes embedded in bundles of collagen fibrils with few accompanying lipid forms (see Fig 9 in Reference 8).

As in previous studies, most of the neutral lipid in the extracellular space was in the form of droplets <0.4 μm in profile diameter. The contrast between the sizes of cellular and extracellular neutral lipid droplets is illustrated in Fig 7. Larger round and scalloped extracellular droplets were somewhat more common in areas in which foam cells were numerous. Very large neutral lipid structures, previously termed “lakes,” had irregular margins and were usually >6 μm. These lakes

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**Fig 2.** Photomicrographs showing immunochemical identification of (A) macrophages and (B) smooth muscle cells, similar to Fig 1, in a human aortic fibrolipid lesion. Arrowhead marks the upper boundary of the musculoelastic intimal layer. The immunostaining patterns resemble those found in fatty streaks (streptavidin-biotin peroxidase technique with hematoxylin counterstain for nuclei, original magnification x150; bar=50 μm).

**Fig 3.** Low-power electron photomicrograph of an early core region, marked by cholesterol clefts, in a lesion with the gross appearance of a fatty streak. A band of elastic tissue, termed the inner limiting elastic membrane, appears diagonally in the center of the photomicrograph. It forms the boundary between the proteoglycan-rich (upper) and musculoelastic (deeper) intimal sublayers. The locations of foam cells above this boundary and cholesterol clefts below it, as illustrated here, are typical for small core regions (osmium–tannic acid–paraphenylenediamine, original magnification x2250; bar=5 μm).

**Fig 4.** Photomicrograph showing extracellular vesicular lipid deposits, demonstrated as round structures with mostly clear interiors, in the core region of a fibrolipid lesion. The dark-staining fibrillar structures are elastic fibers. Numerous collagen fibrils are also present, but no cells are evident in this field (osmium-tannic acid-paraphenylenediamine, original magnification x11 000; bar=750 nm).
FIG 5. High-magnification view of extracellular, mostly vesicular lipid deposits from the core of a fibrolipid lesion. Many of the vesicles have multilamellar structure (arrowhead). Oily droplets of nonpolar lipid are demonstrated as round structures with homogeneous gray staining (arrow). Vesicles are composed of phospholipid and unesterified cholesterol; their presence correlated with chemical analysis showing high levels of unesterified cholesterol, while the presence of droplets correlated with increased cholesteryl ester (osmium–tannic acid–paraphenylene diamine, original magnification ×61,200; bar=140 nm).

sometimes exhibited thick lamellar folds at their margins, and a continuity of neutral lipid was found with adjacent small neutral lipid droplets in a few instances (Fig 8). These continuities could represent either a process of coalescence of smaller droplets to form the larger structure or a budding of small droplets from the larger lake.

In four fibrous plaques, dense deposits of small to very small (down to 20 nm, the size of low-density lipoproteins [LDL]) neutral lipid droplets were found (Fig 9). These droplets were accompanied by relatively few lipid vesicles. The distinct ultrastructural morphology of these lipid deposits correlated well with chemical analysis, showing high cholesteryl ester content, particularly cholesteryl linoleate (see below).

Scattered calcific nodules were found in the core region, especially in those lesions in which extracellular lipid was extensive and cell disappearance advanced. In some cases (Fig 8) the calcific nodule appeared to be surrounded by a lipid membrane, but it was not clear that all nodules were so enwrapped.

Chemical Analysis

The distribution of cholesterol into its free and esterified forms in fatty streaks and in the cap and core region.
of raised lesions is shown in Fig 10. In fatty streaks an average of 39% of total cholesterol was free (unesterified), and 61% was esterified (comparisons are on a molar basis). In the microdissected cores of fibrolipid lesions, 63% of total cholesterol was unesterified, a fraction that differed significantly from fatty streaks (P=.02). In the larger core regions of fibrous plaques, the relative amounts of free versus esterified cholesterol showed wide variation. In two plaque cores the percentage of free cholesterol was greater than 65%, but in other cases the percentage of free cholesterol ranged from 50% to as low as 5%. The cap regions of fibrolipid lesions tended to have high levels of cholesteryl ester and thus resembled fatty streaks. Caps of fibrous plaques had variable fractions of free-total cholesterol, due in part to the fact that some of them contained very little cholesterol at all.

Smith focused attention on the relative accumulations of cholesteryl oleate and cholesteryl linoleate in atherosclerotic lesions as a likely indicator of cellular versus noncellular accumulation of cholesteryl ester. In three samples of plasma LDLs, we found the fraction of oleate within the oleate+linoleate cholesteryl esters (18:1/[18:1+18:2]) to average 0.32. Much higher ratios were associated with cellular lipid accumulation, such as that which occurs in fatty streaks (Fig 11). The fibrous plaque and fibrolipid core regions showed significantly lower 18:1/[18:1+18:2] fractions than fatty streaks (P<.001 and P=.04, respectively). The cap regions of both types of raised lesions were variable, but they tended to resemble fatty streaks. Of particular interest were the fibrous plaque core regions that showed selective accumulation of cholesteryl ester (represented by the bottom four points in the third column of Fig 10). If this lipid was accumulated by the same mechanism (cellular uptake) found in fatty streaks, then one would expect the ester 18:1/[18:1+18:2] fractions in these four lesions to be high. However, some of the lowest 18:1/ (18:1+18:2) fractions were actually found among these lesions, ranging from 0.27 to 0.47. In contrast, the average 18:1/[18:1+18:2] fraction among 11 fatty streaks was 0.80 (see Fig 11 for individual data points).

Structural and Chemical Correlations

As expected, lipid deposits determined to be vesicle-rich by electron microscopy had high levels of free cholesterol, while droplet-rich deposits were high in cholesteryl ester. Cholesterol clefts were found almost exclusively in vesicle-rich areas, and these same areas yielded high measurements of free cholesterol.

Smith and Slater demonstrated a correlation between density of foam cells in frozen sections and the percent of cholesteryl oleate in the oleate plus linoleate fraction in microdissected material from human aortic intima. We graded the degree of foam cell infiltration in the superficial intima on a scale of 1 through 5 and compared this result with the cholesteryl ester 18:1/ (18:1+18:2) ratio measured in the same region. For fatty streaks and caps of raised lesions combined, the correlation coefficient (r) was 48 (P=.01; data not shown). Within lesion types the trends were the same, although statistical significance was attained only for fibrous plaque caps (P=.02).

One of the most interesting correlations was the appearance of densely packed, small extracellular neutral lipid droplets (Fig 9) in the same four fibrous plaque core regions that had high cholesteryl ester contents and low 18:1/18:2 ratios. A further observation was that the lesion caps for these four lesions contained no or sparse foam cells in each case, while foam cells were more numerous in the caps of three of the five remaining fibrous plaques that had relatively more free cholesterol in the core. This last observation must be regarded as preliminary, not only because of the small number of lesion caps showing foam cells, but also because only a portion (1-mm slice) of the cap of these large fibrous plaques was examined.

Discussion

This study focused on the early core region found in human aortic fibrolipid lesions, which were identified on the basis of their raised surface contour and small size. The great majority of distinct, small, raised lesions in human aorta possess pathological lipid deposits at two levels: in superficial foam cells and in...
A deep core region. This study showed that both chemically and morphologically the caps of fibrolipid lesions resemble fatty streaks but that the cores have a completely different character. The most striking finding in early core lipid deposits is their consistently high unesterified cholesterol content. The cholesteryl esters of the early core region were variable, but the more common pattern had a low oleate/linoleate ratio, resembling the cholesteryl esters found in large fibrous plaques.

An additional result of this study was the definition of a second type of extracellular lipid deposit found in the mature core region of fibrous plaque. These were the dense deposits of very small, oily droplets composed predominantly of cholesteryl esters with a low oleate/linoleate ratio.
Extracellular Lipid Deposition

Evidence for extracellular lipid deposition in atherosclerosis is of several kinds. The first stems from the discovery of extracellular lipid in specific circumstances that seem to preclude an origin from cellular lipid. Examples are the lipid droplets found in the interstices of elastic fibers and bundles of collagen fibrils. The subendothelial liposomai structures found in rabbit aorta within 2 weeks after the onset of cholesterol feeding but prior to monocyte infiltration.

The second kind of evidence comes from a broad range of studies that are beginning to suggest mechanisms for trapping and transformation of lipoprotein lipids in the extracellular space of arterial intima. In this category fall the determination of uniquely elevated levels of LDL in arterial intima compared with other connective tissues in the body, the binding of LDL to proteoglycans and elastin, and the aggregation and fusion of LDL particles to form larger lipid structures in vitro and in arterial tissue.

A third kind of evidence comes from observations on lipid deposits in the putative early core region in fatty streaks and fibrolipid lesions. These deposits tend to be associated with and embedded in the extracellular matrix in the deep intima, sometimes at a considerable distance from the prominent foam cell infiltrates in the upper intima.

A fourth kind of evidence aims at determining differences between the cellular lipid deposits found in fatty streaks and the core lipid deposits of fibrolipid lesions and fibrous plaques. The present results fall mainly in this category. The results may reflect the relative contributions to core lipids from direct extracellular lipid deposition versus cellular lipid accumulation and death. Differences have been found with regard to content of unesterified cholesterol, fatty acyl patterns of cholesteryl esters, and sizes of cholesteryl ester droplets as revealed by electron microscopy. The present study is the first to correlate ultrastructure with chemistry and to define lipid composition and structure in the microdissected core region of small, early raised lesions.

Smith and colleagues and others described the differences in cholesteryl ester fatty acyl patterns between fatty streaks and fibrous plaques more than 20 years ago. The present results confirm that the differences result from the lipid composition of the core and not the cap of raised lesions. The best explanation for the increase in cholesteryl oleate in fatty streaks is as follows. Cholesteryl esters in lipoproteins ingested by foam cells are hydrolyzed in lysosomes; the resulting cholesterol is then reesterified in a cytoplasmic location. The reesterifying enzyme, acylcoenzyme A:cholesterol acyltransferase, exhibits a preference for oleyl coenzyme A, resulting in an enrichment of cholesteryl oleate among the cytoplasmic cholesteryl esters. In contrast to fatty streaks, the fibrous plaque core region has a cholesteryl ester fatty acyl pattern that is similar to that in plasma LDL. This fact suggests that most of the core cholesteryl esters have not undergone cellular processing, thus favoring extracellular transformation of LDL cholesteryl esters to lipid droplet cholesteryl esters.

One possibility is that the foam cells in fibrous plaques may not accumulate cholesteryl oleate to the degree that they do in fatty streaks. Indeed, the cholesterol ester 18:1/18:2 ratios that we determined in the caps of fibrous plaques were variable and in some cases quite low (Fig 11). However, the occurrence of foam cells in fibrous plaque caps is also variable, and we found that the cholesteryl ester 18:1/(18:1 + 18:2) fractions correlated well with the presence and density of foam cells. Smith and Slater also microdissected several large atherosclerotic lesions and found that the relative amount of cholesteryl oleate was higher when foam cells were abundant within a microregion of the lesion. Thus it can be concluded that the chemistry of lipid accumulation in foam cells is the same, whether the foam cells are found in fatty streaks or in fibrous plaques.

Forms of Extracellular Lipid

Using electron microscopy, we have identified two forms of extracellular lipid deposits in the core region of mature fibrous plaques: vesicle-rich and droplet-rich. Unlike droplet-rich areas, vesicle-rich areas often contain cholesterol clefts. Based on the analyses of lipid phases provided by Small and colleagues, vesicles were presumed to contain mostly phospholipid and unesterified cholesterol, while droplets were presumed to represent oily deposits of cholesteryl ester. Ultrastructural and chemical correlations in the present study confirmed that the distribution of free and esterified cholesterol matches these predictions.

The temporal sequence of vesicle-rich versus droplet-rich lipid deposition in the core region was uncertain in previous work because only mature core regions were investigated. The present findings on early core regions in small raised lesions (fibrolipid lesions) clearly show that early core lipid deposits are mostly vesicular and contain high levels of unesterified cholesterol. This result is consistent with previous lipid analyses on whole fibrolipid lesions, which also showed strikingly high concentrations of unesterified cholesterol.

Vesicular lipid deposition in atherosclerosis was first identified by Kruth and colleagues as round filipin-staining structures in human and animal lesions. The vesicular structures, subsequently isolated in the same laboratory, were shown to contain high levels of unesterified cholesterol and, interestingly, a relatively high molar ratio of sphingomyelin to phosphatidylcholine. The mechanism of formation of the vesicular structures is quite uncertain. The present study suggested a possible correlation between the presence of foam cells in the cap of raised lesions and vesicular lipid deposition in the core. Thus foam cells are more consistently present in fibrolipid lesion caps than they are in fibrous plaque caps, and vesicular lipid deposition occurs more consistently in the cores of fibrolipid lesions than in the cores of fibrous plaques. Furthermore, when we looked specifically at those fibrous plaques in which the cores contained mostly droplet lipid (cholesteryl esters) rather than vesicular lipid, the lesion caps contained few or no foam cells. This observation, based on only four specimens, must be regarded as preliminary. Nevertheless, one can speculate that foam cells in the cap ingest and hydrolyze lipoprotein cholesteryl esters and then transfer free cholesterol via efflux from the cell surface to other lipoproteins and membranous structures, which eventually transfer the free cholesterol to vesicular lipid.
deposits deep in the intima. Alternatively, foam cells in the lesion cap might secrete cholesterol ester hydrolases that could act on lipoproteins in the extracellular space. This study identified a distinct type of core lipid deposition, suspected from earlier work, in the form of small, densely packed extracellular droplets of cholesterol ester (Fig. 9). Cholesterol oleate/linoleate fractions within this material were low and similar to the fractions in plasma lipoproteins, suggesting direct secretion of the lipid droplets from lipoproteins without cellular uptake and processing. The small size of the individual droplets is also more consistent with extracellular than with cellular lipid accumulation. As noted above, the fibrous plaques that contained this type of core lipid had few foam cells in the lesion cap, consistent with the notion that a lack of cellular uptake and processing might allow lipoprotein cholesterol esters to reach the core region unimpeded. This explanation does not suffice, however, for the single lesion core that contained 95% esterified cholesterol, since that fraction is much higher than the percentage of esterified cholesterol in plasma lipoproteins. Deposition of both esterified and free cholesterol within foam cells, with subsequent selective removal of free cholesterol via reverse cholesterol transport, might account for the very high fraction of esterified cholesterol.

**Immunocytochemistry**

Immunocytochemical analysis yielded results on paraffin sections that were comparable to recent work. In fatty streaks and raised lesions with small cores, macrophages tended to reside in the superficial intima and smooth muscle cells in the deep intima. In lesions with large core regions, macrophages and smooth muscle cells were found at all levels of the intima in complex arrangements. However, the goal of identifying cells specifically associated with early core formation was hampered by an inability to precisely localize small cores in paraffin sections.

**Lesion Development**

The data obtained in this study were consistent with the notion that raised lesions may develop from preexisting fatty streaks. Specifically, the caps of fibrolipid lesions were quite similar to fatty streaks. In the same fibrolipid lesions, the microdissected cores often highly resembled cores of mature fibrous plaques. By combining these results with the recent finding of core formation in the deep intima of flat aortic lesions resembling fatty streaks, a fairly complete transition sequence is defined.

The fact that core development is an early feature in the atherosclerotic process, beginning in flat aortic lesions and already well-developed in some small raised lesions, raises the possibility that some of the toxic, proliferative, or fibrogenic mechanisms in atherosclerosis could represent direct or indirect cellular responses to core lipids. For example, one can speculate that lipid peroxidation might account for cell loss in the core region; but thus far no study has sought to obtain evidence for lipoprotein oxidation in the early core region in human lesions. The high cholesterol content that is especially prominent in the early core region might itself affect membrane functions in cells. Gleason and coworkers have shown that excess cholesterol can cause calcium influx into arterial smooth muscle cells, an effect that demonstrates sensitivity to the calcium channel-blocking agents diltiazem, verapamil, and nifedipine. Whether the classic atherogenic responses of cell proliferation and matrix protein synthesis could be mediated more or less directly by high tissue cholesterol concentrations remains to be determined.

The principal findings of this study are the following: The chemical and ultrastructural characteristics of cholesterol ester droplets in the core region of small and large raised human aortic lesions suggest a largely extracellular mode of depletion. Core regions in small, presumably early, raised lesions have a high content of lipid vesicles rich in free cholesterol, the origin of which has yet to be explained. The fact that small, raised lesions often have caps that are similar to fatty streaks and simultaneously have deep intimal cores akin to fibrous plaques is compatible with the hypothesis of lesion transition.

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