Quantitative Ultrastructural Analysis of Perifibrous Lipid and Its Association with Elastin in Nonatherosclerotic Human Aorta

John R. Guyton, Thomas M. A. Bocan, and Thomas A. Schifani

Nonatherosclerotic areas in human arteries display an age-related accumulation of cholesteryl ester in the form of small, perifibrous lipid droplets in the deeper intimal layers. We treated human aortic specimens with an osmium-thiocarbohydrazide-osmium sequence en bloc after glutaraldehyde fixation in order to provide electron dense staining of neutral lipid for ultrastructural study. Neutral lipid was quantified in terms of area fractions on thin sections. Extracellular lipid, primarily in the form of small (<300 nm) droplets, accounted for 91% of the lipid found in the deep intimal region. Seventy-four percent of extracellular lipid appeared in droplets or aggregates that were demonstrated as adjacent to or within elastic fibers in the plane of section. The fraction of lipid adjacent to elastin in three dimensions is likely to be considerably higher than 74%. The results support the concept that an interaction between elastin or its associated components and lipids or lipoproteins may be important in extracellular lipid deposition in human arteries.

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In the second decade of life, humans begin to accumulate cholesteryl ester in atherosclerosis-free regions of the intima of large arteries. Microscopically this accumulation takes the form of small extracellular droplets of neutral lipid associated with fibrous tissue elements, i.e., perifibrous lipid. Little attention has been given to the ultrastructural description of perifibrous lipid, probably because of inadequate methods of visualizing it by electron microscopy. An ultrastructural description will give improved insight into extracellular mechanisms of lipid deposition in normal aging arteries and possibly contribute toward an understanding of the predominantly extracellular lipid deposition in fibrous plaque atherosclerosis.

The extracellular space in arterial intima consists of several components, primarily fibrillar collagen, elastin, basement membrane, and reticular ground substance (proteoglycan matrix), which may be distinguished by electron microscopy. An obvious question is whether perifibrous lipid may be randomly distributed within the extracellular space or specifically localized in the vicinity of one of these structural components. Most light microscopic studies have suggested an association between perifibrous lipid and elastin, and some studies have discussed the additional involvement of collagen.

By routine electron microscopy, the small droplets of perifibrous lipid appear as round, lucent areas from which most of the lipid has been extracted by processing. The poor retention and staining of neutral lipid by routine techniques has been the chief deterrent to adequate ultrastructural description of perifibrous lipid. In the course of attempting to enhance lipid identification by several methods, we became aware of a report by Willingham and Rutherford that neutral and membranous lipid in tissue culture cells could be stained for electron microscopy by an osmium-thiocarbohydrazide-osmium (OTO) sequence. They had adapted the technique from an earlier one by Seligman and colleagues. Thiocarbohydrazide (\( 2HNNHCSNHNH_2 \), TCH) apparently acts to enhance the staining properties of osmium tetroxide, by bridging osmium originally fixed in the tissue to additional osmium provided after TCH.

We have applied this technique to grossly normal aortic tissue from 18 human autopsy cases, presenting a range of intimal deposition of perifibrous lipid. In...
addition to qualitative observations, we have quanti-
ified the area fractions, sizes, and location of extra-
cellular lipid as revealed in thin sections prepared for
electron microscopy. The results disclose a consist-
tent pattern of intimal involvement, with a predomi-
nant association between lipid and the surfaces and
interior of elastin.

Methods

Specimens and Tissue Samples

Aortic specimens were obtained from autopsies of
18 individuals (11 males and seven females) aged 6
to 66 years. Most deaths were due to trauma. Speci-
mens were obtained approximately 18 hours after
the time of death, with the body kept in a 4°C room
for most of this time.

Sampling sites were chosen with the goal of de-
monstrating a spectrum of intensities of perifibrous lip-
id deposition. From each case one area of grossly
normal, flat intima was selected from the descending
thoracic or upper abdominal aorta, avoiding sites ad-
Jacent to orifices. Frozen sections were cut at 10 /m
and stained with 1.0% Oil Red O in 60% isopropanol.

Primary fixation of 1 mm cross-sectional slices for
electron microscopy was carried out in 3% glutara-
dehyde in 0.1 M sodium cacodylate buffer (pH 7.4)
for 24 hours at 4°C. This tissue was subsequently
processed by several methods, including the OTO
sequence described by Willingham and Rutherford.5
Preliminary work showed that tissue penetration by
this procedure was limited to a depth of approxi-
mately 150 micrometers. Therefore, following primary
fixation, all tissue slices were further sectioned at 100
to 200 micrometers using a Vibratome Tissue Sec-
tioner. The preferred method (OTO) for secondary
fixation and staining en bloc was as follows: incuba-
tion for 30 minutes in 2% aqueous OsO4, extensive
washing with four buffer changes of 3 minutes each,
and repeat washing in maleate buffer (pH 5.2). All
sections were washed and stained with t.0% Oil Red O
in 60% isopropanol.

For electron microscopy, the first complete elastic
lamina in the intima (inner limiting membrane) defines the lower
boundary of the musculoelastic (deepest) intimal lay-
er. The first complete elastic lamina in the tissue section, then posi-
tioned the first sampling micrograph at the midpoint
by averaging the coordinates. Thus, the first micro-
graph was not precisely aligned with the elastic lam-
ina, but always included it. Two additional micro-
graphs were then taken, one 50 /m toward the lumen and one 50 /m toward the adventitia.

Tissue slices were dehydrated at 4°C in a graded
acetone series to 100%, placed in 1:1 acetone:
Epon 812 overnight, and embedded in Epon 812.
Thin sections were cut on an LKB Ultratome III and
viewed with a JEOL 200CX electron microscope at
80 kV. No section staining was necessary for speci-
mens prepared with thiocarbohydrazide. Sections
from routinely prepared samples were stained with
uranyl acetate and lead citrate.

Micrograph Sampling Strategy and
Morphometry

There were two goals in determining locations for
electron micrographs. The first was to identify the
region known by light microscopy to have the great-
est deposition of lipid, i.e., the vicinity of the inner
boundary of the musculoelastic (deepest) intimal lay-
er. The first complete elastic lamina in the intima
(inner limiting membrane) defines this boundary. To-
ward the luminal side of this boundary, elastin is
present as fragmented structures. The second goal
in sampling was to avoid unaccounted bias in deter-
mining locations for individual micrographs. To do
this, we read x,y coordinates from the microscope
stage micrometers at the cut edges of the first com-
plete elastic lamina in the tissue section, then posi-
tioned the first sampling micrograph at the midpoint
by averaging the coordinates. Thus, the first micro-
graph was not precisely aligned with the elastic lam-
ina, but always included it. Two additional micro-
graphs were then taken, one 50 /m toward the lumen and one 50 /m toward the adventitia.

The extent of this distance and variability of spacing
between elastic membranes are such that bias for or
against including elastin in these latter micrographs
can be considered minimal. Micrographs were print-
ed at a magnification of 14000 for morphometry.

Morphometric analysis embodied three aspects —
lipid quantification, lipid localization, and quantifi-
cation of major structural elements. We required that
"visible" lipid have the appearance of round droplets
or aggregated droplets, each droplet either homoge-
eous or bounded by a thick rim with an electron
density greater than that of any nonlipid and nonmin-
neral structure. Difficulty in applying this criterion was
encountered only rarely. Based on a sample distribu-
tion of 380 droplet profile sizes, it appeared likely that
droplet profiles of less than 30 to 50 nm diameter
were rarely recognized. Thus, individual low density
lipoprotein (LDL) species were excluded from recog-
nition, although this lipoprotein may have been pres-
ent within some of the lipid-rich aggregates noted.

A significant issue for stereologic quantification by
point counting is the dual question of overestimation
of volume fraction due to the Holmes effect in sec-
tions of finite thickness, as well as the underestima-
tion due to lack of recognition of lipid that traverses
the section thickness incompletely. To gauge the
magnitude of these potential errors, we used an ap-
proximate solution offered by Weibel.10,11 The diam-
ers of 380 randomly selected lipid droplet profiles
from 18 micrographs were scaled, using 12 size
ranges. The mode of profile diameters was in the range of 70 to 105 nm, approximately the thickness of our sections, and the distribution was highly skewed by the presence of small numbers of profiles up to four times this diameter. Because of this skewness, Weibel's procedure could not be followed exactly, since profile diameter frequencies to the left of the mode could not be corrected for section thickness by reflecting frequencies from the right side of the mode. We considered instead that numbers of profiles, with diameters of less than one section thickness, would be overestimated approximately in inverse proportion to their diameter. Assuming a section thickness of 80 nm and assuming recognition of lipid extending through at least 30% of section thickness (this assumption is consistent with the lower limit of profile diameters) and using the method of Cruz-Orive as described by Weibel to obtain a distribution of particle diameters, we calculated the final correction factor to be 0.77 (volume fraction = 0.77 times area fraction). All considered, this result was simply taken as reassurance that estimates of volume fractions would not be greatly in error. Lipid quantities are reported as uncorrected area fractions.

The second phase of morphometric analysis was to provide estimates of the relative amounts of lipid located within or adjacent to various structural elements. This was limited to a two-dimensional analysis. We recorded locational information in the microcomputer memory at the same time that point counting for lipid quantification was performed using a square grid overlay of 3672 points per micrograph. For each intersection between the point grid and a lipid deposit, we keyed codes for any structures adjacent to a structure if the droplet itself lifted away by other droplets or other types of lipid-rich material formed beneath it, we counted the droplet as adjacent to a structure if the droplet itself touched (was within 55 nm of) the structural surface or if it was in a lipid-rich aggregate structure that touched the surface. Aggregate structures considered in this way were never larger than 600 nm. The expressed result is the fraction of total lipid (that is, area fraction) that appeared adjacent to any given structure within the plane of section. This fraction may be appropriately termed a "cross-sectional adjacency fraction."

To complete the analysis of lipid localization, it was necessary to obtain the overall area fractions of tissue structural elements. This was done by point counting with a much coarser grid overlay, approximately 220 points per micrograph. Because these structural regions had dimensions generally much larger (fivefold or more) than section thickness and because regions were identified both by the presence and absence of electron dense staining, the Holmes effect was disregarded, and area fractions could be assumed to estimate volume fractions (the Delesse principle).

Structural regions were categorized as follows: fibrillar collagen consisted of distinct fibrils with a banded appearance on tangential section. Elastin formed layers and seams with a typical glassy homogeneous appearance, containing streaks of increased electron density. In some places elastin was arranged in incomplete layers and coarse fibers, which showed a fibrillar substructure. Areas showing distinct microfibrils were scant; these areas were included with elastin form morphometry. Basement membrane was identified as one or more layers of material of intermediate electron density and of variable width, adjacent to smooth muscle cells, and having a homogeneous appearance within each layer. Reticular ground substance comprised the electron lucent areas not otherwise occupied by distinct structures, but which did contain sparse granules and fine fibrils in a reticular pattern. This space is thought to be filled by a loose meshwork of proteoglycan aggregates. Cells encountered in the deeper layers of the aortic intima were predominant-

<table>
<thead>
<tr>
<th>Level</th>
<th>Fibrillar collagen</th>
<th>Elastin</th>
<th>Basement membrane</th>
<th>Reticular gnd. subst.</th>
<th>Cells</th>
<th>Other</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0.34† (0.24)†‡</td>
<td>0.72 (0.22)</td>
<td>0 (0.09)</td>
<td>0.57 (0.26)</td>
<td>0 (0.15)</td>
<td>0.01 (0.04)</td>
</tr>
<tr>
<td>2</td>
<td>0.17 (0.28)</td>
<td>0.87 (0.30)</td>
<td>0 (0.04)</td>
<td>0.36 (0.21)</td>
<td>0 (0.09)</td>
<td>0 (0.08)</td>
</tr>
<tr>
<td>3</td>
<td>0.30 (0.27)</td>
<td>0.76 (0.27)</td>
<td>0 (0.08)</td>
<td>0.44 (0.12)</td>
<td>0 (0.21)</td>
<td>0.03 (0.05)</td>
</tr>
</tbody>
</table>

For comparison, structural area fractions are given in parentheses.

*Level 1 was centered 50 μm luminal from the inner limiting elastic membrane (ILEM), or if <50 μm, just beneath the lumen; Level 2 at the ILEM; and Level 3, 50 μm abluminal from the ILEM.
†Cross-sectional adjacency fraction, representing area of extracellular lipid droplets or aggregates adjoining the designated structural component, divided by total area of extracellular lipid on micrographs.
‡Area fraction of structural component, shown in parentheses, representing component area divided by total micrograph area.
ly smooth muscle cells. To assess reproducibility of structural area estimates, duplicate point counts were performed on 11 micrographs. The standard deviation for replicate counts (square root of variance portion) ranged from 2% to 4% of total points counted. Standard errors for the structural area estimates given in Table 1 ranged from 0.01 to 0.04 (n = 18).

We made a statistical comparison of adjacency fractions by analysis of variance, using only those specimens containing substantial lipid (n = 14) and using the Bonferroni multiple comparisons procedure to correct the p value. 12

Results

Light Microscopy

Total intimal thickness for the human aortic specimens ranged from 25 to 350 μm, generally increasing with age. Oil Red O staining of frozen sections by design revealed a spectrum of lipid deposition, increasing with advancing age and intimal thickness. Most of the Oil Red O-stained lipid was in the form of small (less than 0.5 μm) droplets and appeared to be extracellular. Some of the deposited lipid simply gave a diffuse red color to the tissue, indicating that sizes of individual deposits were below the limit of light microscopic resolution (0.2 μm). Often the largest Oil Red O-stained droplets in a given area came into clear focus just above or below the tissue section, indicating an artifactual coalescence of tissue lipid. In the great majority of specimens, foam cells packed with lipid were absent, as indicated by a lack of clustered, large droplets.

The location of lipid staining in various specimens could be categorized into three patterns (Figure 1). Most commonly, lipid accumulated within and surrounding the inner limiting elastic membrane, which marks the inner boundary of the musculoelastic (deepest) intimal layer. Adjacent elastic membranes also showed lipid accumulation. With heavier deposition, lipid staining extended into other interstitial tissue and deeper into the musculoelastic layer as far as the internal elastic lamina. In a second pattern found in only two specimens from subjects in their twenties, scattered intimal foam cells as well as extracellular lipid extended from the lumen surface through the intima and intimal layers to the inner limiting membrane, sparing the musculoelastic layer. In the third pattern, dense lipid accumulation was found throughout the intima, with a few large, apparently intracellular deposits as well as abundant small extracellular droplets.

Electron Microscopic Appearance of Lipid

Routine processing of tissue for electron microscopy (Figure 2) neither stained extracellular lipid well nor stabilized it against extraction by solvents used for dehydration. Thus, identification was made when a clear vesicle, sometimes with a crescent of homog-

Figure 1. Patterns of lipid deposition in grossly normal human aortic intima, demonstrated in 10 μm transverse frozen sections stained with Oil Red O. Lumen is at the top. Arrowheads are at the internal elastic lamina. The inner limiting elastic membrane (ILEM), which marks the inner boundary of the musculoelastic intimal layer (MEL), is approximately in the middle of each micrograph. A. Common lipid pattern with involvement of MEL, especially near ILEM. B. Unusual pattern, showing lipid deposition from lumen to ILEM, with subendothelial foam cells. C. Heavy lipid deposition, mostly in the form of fine droplets, extending throughout intima. Hoffman modulation contrast optics, × 265. Bar = 50 μm.

Figure 2. Electron micrograph of routinely processed tissue showing lipid droplets mostly within elastic membranes. Note that the interiors of droplets appear almost entirely extracted by solvents during processing. Droplets cannot be distinguished from vesicles with aqueous centers. × 20,900. Bar = 0.5 μm.
Figure 3. OTO-stained specimen showing dense accumulations of neutral lipid within and adjacent to elastin. Occasional droplets have a “cut-out” or partially collapsed contour, possibly artifactual (arrow). Note the absence of lipid in basement membrane adjacent to cells and in the open spaces (reticular ground substance) between formed elements. The smooth muscle cell at the upper left contains an electron dense intracellular lipid droplet. × 12,600. Bar = 1 μm. Inset: Higher magnification of lipid droplets associated with elastin, showing thick, dense rim of osmium surrounding larger droplets. × 23,200. Bar = 0.5 μm.

From the calculated distribution of spherical particle sizes, it was estimated that one-half or more of the total extracellular lipid content was contained in droplets with individual diameters less than 180 nm. Extracellular droplet diameters ranged as high as 400 nm. Most commonly, droplets appeared individually or, if aggregated, simply packed together according to the constraints of the tissue in which they appeared (i.e., in a seam within amorphous elastin, as in Figure 3). At times aggregates of lipid were found external to elastin (Figure 4 A). A more problematic appearance is that shown in Figure 4 C which occurred in several specimens. In these cases a relatively broad area of the section was filled with mostly spherical gray structures of intermediate electron density. Within this area distinct droplets of neutral lipid were found. The gray spheres often had an electron density greater than that of membranous vesicles and thus contained an osmiophilic, possibly lipid-rich, interior. However, they did not meet our criteria for definite neutral lipid. As with definite neutral lipid, these structures were often seen to border on elastin.

Lipid Quantification and Localization

The area fractions for extracellular lipid in individual micrographs ranged from 0 to 3.4%. Values for the three micrographs in each specimen were averaged, and the relationship to subject age is shown in Figure 5. When average values were calculated by depth levels, the following results were obtained: For micrographs taken 50 μm, or as far as possible, to the luminal side of the inner limiting elastic membrane (Level 1), the lipid area fraction averaged 0.6%, for micrographs containing this elastic mem-
Figure 4. Other appearances of perifibrous lipid. **A.** OTO-stained specimen showing heavy lipid deposition appearing as aggregates. Most of the elastin in this micrograph was considered to have the fibrillar, or immature form. × 14,000. Bar = 1 μm. **B.** Heavy lipid deposition associated with elastin and fibrillar collagen. Note the tendency to find lipid in holes within these structures. × 12,600. Bar = 1 μm. **C.** Probable lipid deposition with an intermediate level of staining by OTO. A broad band (large arrows) of aggregated spherical gray structures appears next to an elastin lamina. Although this mass of material may have a high lipid content, only a few droplets (arrowheads) were dark enough to be counted definitely as lipid for quantitation. A few vesicles are present (small arrows). Note the lack of involvement of basement membrane next to smooth muscle cells at lower right. × 10,400. Bar = 1 μm.

brane (Level 2), 1.1%, and for micrographs 50 μm toward the adventitial side of the membrane (Level 3), 0.7%. The peak at the elastic membrane is due in part to selection bias for elastin, which was found to be highly associated with lipid. It must be recognized that these micrographs are not a representative sampling of the entire intima, but only of the deeper layer (especially in older individuals with relatively thick intima), where perifibrous lipid is most likely to be found.

The cross-sectional adjacency fractions in Table 1 verify quantitatively what may be seen in virtually any micrograph, that is, the marked tendency for lipid to

Figure 5. Relationship between perifibrous (extracellular) lipid area fractions and age. The results from three micrographs for each specimen were averaged.
be associated with the surfaces and interior of elastin. Taking only Levels 1 and 3, which were not locally biased to include elastin, we found that approximately 74% of the extracellular lipid was associated with elastin. The cross-sectional adjacency fractions showed elastin > reticular ground substance > collagen > other structures, with each inequality significant (p = 0.01 or less). The fact that the sum of cross-sectional adjacency fractions at a given level exceeds unity is due to the many lipid droplets situated at interfaces between structures. One should note that Table 1 categorizes extracellular lipid only. The adjacency fraction of zero for cells means that no extracellular lipid appeared adjacent to a plasma-lamellar membrane.

Table 1 also shows in parentheses the area fractions of structural components. The higher fraction of elastin at Level 2 compared to Level 3 is due to selection for elastin in the former. By comparing lipid adjacency fraction to the corresponding structural area fraction, an indication of the specificity of lipid deposition on or in a particular structure gained. Based on this comparison, one must consider that the reticular ground substance as well as elastin may play a role in lipid deposition.

Despite the comparatively low lipid adjacency fraction for collagen, we did on occasion find clear evidence of droplets within collagen bundles (Figure 4 B). Particularly when the collagen was cut in cross section, it seemed quite unlikely that these droplets were actually in contact with elastin out of the plane of section. The few specimens with a definite lipid-collagen association were all heavily infiltrated with lipid. With increasing lipid infiltration, both elastin and collagen appeared to develop interior holes and gaps of increasing size, within which lipid was found (Figure 4 B).

Intracellular lipid constituted only 9% (as an area fraction) of the total lipid quantified in this study. Intracellular lipid droplets, often more than 500 nm in profile diameter, were much larger than those found extracellularly. Most of the intracellular lipid was found in smooth muscle cells, identified by pericellular basement membrane, myofilaments, dense bodies, and pinocytic caveolae. It should be recognized that we did not examine quantitatively the more superficial intima in older individuals. On viewing the specimens qualitatively, we found occasional foam cells near the lumen, possibly derived from macrophages, possessing micropodia and no basal lamina.

**Discussion**

The most important conclusion from our study relates to the prominence of elastin as a site for lipid deposition in aortic intima of adults. Confidence in the identification of lipid droplets by electron microscopy was afforded by use of OTO after glutaraldehyde fixation. The patterns of lipid deposition in and around elastin, and sometimes within collagen and other structures, give insight into potential mechanisms for lipid deposition and the evolution of intimal pathology.

**Usefulness of OTO Procedure**

Our results clearly demonstrate the applicability of OTO as an en bloc procedure for the identification of lipid in tissue sections. Osmium has long been used as a lipid stain in light microscopy. In the initial application of the OTO sequence by Seligman and colleagues, the thiocarbohydrazide and second osmium tetroxide steps were performed on thin sections of plastic-embedded tissue. The intense staining reaction of lipid is thought to depend upon its density of osmiophilic sites. In our work, lipid identified ultrastructurally by OTO staining corresponded in relative amounts and location with that shown by light microscopy with Oil Red O. Chloroform pretreatment removed OTO stainable lipid. Importantly, the electron density of lipid after OTO treatment was clearly retained in the tissue much better than that after osmication alone. This provided assurance that major sites of lipid deposition were not being eliminated by solvents. The limited penetration by OTO into droplets larger than 100 to 200 nm led to a highly characteristic doughnut-shaped appearance, the interior of which retained considerable electron density. Vesicles with apparently aqueous centers had a narrow membranous rim and nonstaining interior, and thus were easily distinguished from lipid droplets. Nevertheless, for the smallest presumed lipid deposits and for certain structures with granular texture or intermediate electron density, considerable uncertainty remains.

**Perifibrous Lipid**

Our observations on the light microscopic appearance of grossly normal aortic intima, stained by Oil Red O, conform to previous observations. Smith and colleagues described perifibrous lipid as sudanophilic droplets 0.5 to 1.5 μm in diameter, associated particularly, but not entirely, with fragmentary elastic laminae in the musculoelastic intimal layer. Our ultrastructural observations suggest that the size range for the lipid droplets is actually much lower. The discrepancy might be explained by the perception of lipid aggregates as droplets by light microscopy and by the apparent artificial coalescence of lipid due to solvents used in staining with sudanophilic dyes.

The age-related increase in perifibrous lipid area fractions, beginning at about age 20 (Figure 5), closely parallels the rise in cholesteryl ester content of normal intima shown by Smith. We can ask whether this postulated identity between increasing cholesteryl ester and perifibrous lipid is reasonable in quantitative terms. The area fractions for perifibrous lipid ranged between 0.7 and 2.2% in older individuals. For reasons given earlier, the corresponding volume fractions are probably similar. Pre-
vously we have found cholesteryl ester content of nonatherosclerotic aortic intima in comparably aged persons to average 1 mg/100 mg dry defatted weight, a result consistent with other work. Considering tissue wet weight to be three- to fourfold higher than dry defatted weight and intimal density and cholesteryl ester density close to 1 g/ml, we estimate that the average volume fraction for cholesteryl ester in the entire intima is approximately 0.3%. This is quite compatible with our somewhat greater area fraction observed in the lipid-rich deeper portion of the intima.

The importance of perifibrous lipid as the major site of cholesteryl ester deposition in grossly normal aortic intima is further supported by several observations, especially from the extensive studies of Smith and colleagues. Morphologic-chemical correlations between perifibrous lipid and cholesteryl ester levels have been established for intimal sublayers and for individual pairs of adjacent tissue specimens. The concentration of apparently intact LDL or apolipoprotein B based on electroimmunoassay is much too low to account for the increasing cholesteryl ester. With regard to extracellular versus intracellular cholesteryl ester deposits, the electron microscopic observations in the present study, including qualitative observations extending to the luminal surface, concur with previous light microscopic judgements that the majority of neutral lipid in normal intima is extracellular rather than intracellular. Moreover, Smith has shown that the pattern of fatty acyl groups esterified to cholesteryl in normal intima from middle-aged and older persons is typical of that found in extracellular, but not intracellular cholesteryl ester.

**Location of Lipid**

The two-dimensional analysis of extracellular lipid location suggested that at least 74% of lipid within the deep intimal region was intimately associated with elastin. It is likely, however, that the actual three-dimensional fraction is considerably higher, because the area of contact between lipid or lipid aggregate and elastin would be expected to occur out of the plane of section for many such associations. The section profiles of lipid droplets were often seen just out of contact with a tangentially sectioned edge of elastin. Moreover, when lipid accumulation was dense, small amounts of elastin could easily have been missed. Thus, the evidence suggests that elastin is the dominant site for perifibrous lipid deposition in human aorta.

Several workers (particularly Kramsch, Hollander, and Franzblau) suggested a specific role for elastin in arterial lipid accumulation. Using autoradiography, these authors have demonstrated localization of injected $^3$H-cholesterol in elastic membranes. Furthermore, elastase treatment was found to release considerable quantities of cholesterol from normal aortic intima and atherosclerotic plaque. Kramsch and Hollander, and others found that large amounts of cholesterol derived from LDL bind to elastin after a relatively brief (24-hour) incubation. Noma and colleagues extended this work by demonstrating immunologically the presence of apoprotein B on the surface of LDL-incubated elastin.

Substantial proportions of extracellular lipid appeared adjacent to or within the reticular ground substance and fibrillar collagen in this study. However, it is obvious from Table 1 that much of this lipid actually was located at the interface between these structures and elastin. The potential roles of the reticular ground substance and fibrillar collagen in lipid deposition are, therefore, less clearly demonstrated than the role of elastin. There is evidence that certain proteoglycans may bind lipoproteins in the arterial intima. One should note that proteoglycans are found not only in the reticular ground substance between collagen and elastin, but also in close association with these fibrous proteins. With high levels of lipid deposition, we found evidence that some lipid droplets form completely within bundles of collagen fibrils (Figure 4 B). This phenomenon has a parallel in extravascular tissue, where cholesteryl ester deposits have been found in the extracellular space in tendons.

The implications of lipid or lipoprotein interaction with extracellular tissue components are still unclear, but it is interesting to speculate whether there may be an upper limit to lipid infiltration of the intima, beyond which the metabolic function of the tissue is disturbed. Recently we have found evidence to suggest that the formation of the lipid-rich core region of the atherosclerotic fibrous plaque may be a very early event, occurring in small lesions with minimal elevation. In the smallest such lesions, the core consisted of extracellular lipid found in the musculoelastic layer of the intima, a location similar to that of perifibrous lipid. With increasing extracellular lipid deposition and larger volume of the core region, elastin may decrease as a morphologically recognizable entity, at a time when collagen is relatively preserved (Bocan TMA, Schifani TA, and Guyton JR, unpublished observations). It is possible that diverse observations can be tied together eventually into a mechanism of core region development involving elastin-lipoprotein interaction and cellular responses.

**Acknowledgments**

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