Compartmentalization of Water in Human Atherosclerotic Lesions

Changes in Distribution and Exclusion Volumes for Plasma Macromolecules

Elspeth B. Smith and Christopher Ashall

High concentrations of LDL and other plasma macromolecules are present in normal aortic intima and early proliferative atherosclerotic lesions that have not yet accumulated lipid. Their compartmentalization within the tissue water was estimated from the concentrations of low density lipoprotein (LDL), α2-macroglobulin (α2-M), and albumin in interstitial fluid and adjacent intimal tissue. Interstitial fluid was collected on filter paper inserted into natural strip planes in the intima; macromolecules in interstitial fluid and tissue were assayed by rocket immunoelectrophoresis directly from the papers or tissue. From their concentrations in interstitial fluid and total tissue water, the fractional and absolute volumes in which they were distributed (distribution volume) or from which they were excluded (exclusion volume) were calculated for each macromolecule. Compared with normal intima, the distribution volume increased by 60% and the exclusion volume by 95% in early gelatinous lesions; exclusion volume fraction was linearly related to molecular mass. In more advanced lesions, there was disproportionate exclusion of LDL and α2-M. Their distribution volumes decreased and their concentrations in interstitial fluid increased; the concentration showed a significant inverse relation with distribution volume (regression coefficient, b = -0.53, p < 0.01). Redistribution of tissue water may concentrate LDL in interstitial fluid, and contribute to its extracellular deposition.

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The immediate nutrient environment of the cells in all tissues is provided by the interstitial fluid (IF). It has long been assumed that change in the environment of arterial smooth muscle cells, particularly in the concentration of low density lipoprotein (LDL) is a major factor in atherogenesis. Recently, we developed a method for collection of IF from human aortic intima, and measured the concentration of three plasma macromolecules — LDL, α2-macroglobulin (α2-M) and albumin — by rocket immunoelectrophoresis.1,2 Surprisingly, in IF from normal intima the concentration of LDL was more than twice the concentration in the patient’s own plasma.

The study of isolated IF allows analyses of freely diffusible plasma macromolecules independently of possible interactions with components of the intimal connective tissue matrix. Furthermore, direct comparison of IF and adjacent intact intimal tissue provides a method for investigating specific reversible binding, as this should give rise to different ratios between the macromolecules in IF and tissue.1,2

It is obvious on gross inspection and dissection of atherosclerotic lesions that there are wide variations in water content. The soft, proliferative gelatinous lesions3 contain a large amount of fluid that tends to leak out of the lesion during dissection, whereas the white caps of fibrous plaques appear rather dry. Total tissue water can be obtained from wet and dry weight measurements; if we also measure the concentrations of macromolecules in IF, and the amounts in adjacent intimal tissue, we can calculate...
the proportion of the total tissue water that contains the macromolecules (the distribution volume) and the proportion from which they are excluded (exclusion volume). This provides additional insights into the compartmentalization of water and changes that occur during lesion development.

In this study we have examined only early proliferative lesions in which there is no excess of lipid, and the low-lipid, white collagenous caps of plaques. The reasons for excluding lipid-rich lesions are threefold. In the vicinity of fat-filled cells in fatty streaks, LDL is depleted in both IF and tissue, apparently as a result of uptake and degradation by the fat-filled cells. Papers for collection of IF that were inserted into areas containing abundant extracellular lipid might become contaminated with the lipid, and this would invalidate the measurements of IF mass. We do not know how to calculate exclusion due to lipid.

**Methods**

**Aortas**

Aortas were obtained between 5.5 hours and 28 hours after death (mean 16.7) from seven men and nine women from whom a blood sample had been taken for routine analysis during the 3 days before death. The men were aged between 43 and 77 years (mean 63) and the women, 41 to 74 years (mean 61). One sample was obtained within a few minutes of death. The men were aged between 43 and 77 years (mean 63) and the women, 41 to 74 years (mean 61). One sample was obtained within a few minutes of death.

**Collection of Interstitial Fluid**

The method of collection of IF has already been described in detail. Briefly, Whatman No. 1 filter paper was cut into pieces measuring 2 x 2 mm (for measurement of albumin) and 2 x 3.5 mm (for measurement of LDL and \( \alpha_2 \)-M) and weighed on an electronic balance. To avoid variable uptake of atmospheric moisture during insertion, the papers were freed of adherent agarose, extracted with ethanol/ether (3:1) followed by methanol/chloroform (1:1) for 48 hours to remove lipid, removed from the papers, dried to constant weight at 37°C, and weighed.

**Measurement of Plasma Proteins**

The amounts of LDL, \( \alpha_2 \)-M, and albumin in IF and tissue samples were measured by quantitative rocket immunoelectrophoresis; papers carrying IF and parcels of intimal tissue were embedded in adjacent positions in agarose plates and the antigens were electrophoresed directly into antibody-containing gels as described previously. LDL and \( \alpha_2 \)-M were assayed simultaneously in the same sample, the antigens migrating first into a window of antiserum to \( \alpha_2 \)-M and then into antiserum to LDL. For quantification, samples of the patient’s own serum (PS) and of a standard calibrated plasma were run on each plate; the amount of antigen can be calculated in absolute terms from the standard plasma, or in terms of equivalent microlitres of the patient’s serum. In IF, when this is expressed as a concentration — \( \mu \)l PS/100 mg IF — it is virtually identical to the percentage of serum concentration.

**Reproducibility of Quantitative Rocket Immunoassay**

Mean differences among duplicate samples of IF from eight areas of normal intima were 6.0% for LDL and 7.0% for \( \alpha_2 \)-M and albumin. Within-plate variation for the standard plasma was ± 1.3% for LDL (53 pairs), ± 2.4% for \( \alpha_2 \)-M (38 pairs), and ± 2.6% for albumin (23 pairs).

**Results**

From the wet and dry weights of the tissue we can obtain the weight of the total tissue water and thence the concentration of the tissue macromolecules in the total tissue water. If this is compared with the concentrations in the interstitial fluid, we can calcu-
late the proportion of the total water that is in the IF compartment, and hence its volume. We have called this the "distribution volume" and the remaining water, the "exclusion volume"; the latter is presumably intracellular water and water associated with collagen fibers, glycosaminoglycans, and other components of the connective tissue matrix that exclude macromolecules. In the calculation it is assumed that all the LDL in tissue is either free or in a tightly bound form that is not dissociated and extracted in the electric field. Direct comparison of the ratios of LDL to $\alpha_2$-M and albumin in IF and tissue suggests that this assumption is justified.

**Total Tissue Water, and the Concentrations of Macromolecules In Interstitial Fluid**

The total water content and the concentration of plasma macromolecules in IF were compared in normal intima and proliferative atherosclerotic lesions (Table 1). The gelatinous lesions were soft, translucent thickenings containing little or no stainable lipid. Both the average total water content and the concentrations of LDL and $\alpha_2$-M in the IF were significantly higher than in normal intima ($p < 0.01$), but there was no significant change in the concentration of albumin.

The lesions that we have called "transitional" were soft elevations that appeared grayish or slightly opaque, and were assumed to represent the next stage in the evolution of gelatinous lesions into fibrous plaques; they also contained little stainable lipid. Compared with the gelatinous lesions, their water content was significantly lower ($p < 0.001$). The IF from some of these lesions contained the highest concentrations of LDL and $\alpha_2$-M that we encountered. However, they appear to be a heterogeneous group and probably form a continuum with the gelatinous lesions. The distributions of LDL concentration in IF from gelatinous and transitional lesions are compared in Figure 1.

In this study, only normal intima and proliferative lesions with no lipid staining, pale, diffuse sudanophilia, or small amounts of fine, perifibrous lipid have been included; all samples containing intracellular lipid droplets or abnormal accumulations of extracellular lipid were excluded. The white fibrous plaques had lipid-rich centers, but only the low-lipid collagen caps were analyzed. Their water content was significantly lower than normal intima or transitional plaques ($p < 0.01$). The concentration of LDL in IF was about one-half that in IF from normal intima ($p = 0.001$), but $\alpha_2$-M and albumin were not significantly different. There were no significant differences between the concentrations of albumin in IF from normal intima and any of the atherosclerotic lesions.

### Table 1. Total Tissue Water, and Concentration of Plasma Macromolecules in Interstitial Fluid (IF) from Normal Intima and Atherosclerotic Lesions

<table>
<thead>
<tr>
<th>Lesion</th>
<th>Total tissue water mg/100 mg dry tissue</th>
<th>IF collected: mg/100 mg paper</th>
<th>Concentration in IF % of plasma concentration</th>
<th>Maximum thickness μm</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal intima (n = 11)</td>
<td>766 ± 52</td>
<td>176 ± 11</td>
<td>LDL 226 ± 15, $\alpha_2$-M 330 ± 35, Albumin 118 ± 26</td>
<td>233 (100–350)</td>
</tr>
<tr>
<td>Gelatinous thickenings (n = 11)</td>
<td>1257 ± 91</td>
<td>194 ± 8</td>
<td>711 ± 63, 432 ± 98, 118 ± 26, 93 ± 13</td>
<td>951 (210–2450)</td>
</tr>
<tr>
<td>Transitional plaques (n = 9)</td>
<td>711 ± 91</td>
<td>168 ± 9</td>
<td>1455 (350–3350)</td>
<td></td>
</tr>
<tr>
<td>White caps of plaques (n = 6)</td>
<td>493 ± 42</td>
<td>184 ± 6</td>
<td>2150 (1550–3000)</td>
<td></td>
</tr>
</tbody>
</table>

Values are means ± SEM; parentheses indicate range of thickness.

### Compartmentalization of the Tissue Water

The volumes of tissue water in which the macromolecules were distributed (distribution volume) and from which they were excluded (exclusion volume) are shown in Table 2.

The distribution volume was inversely, and exclusion volume directly, related to molecular mass. In the gelatinous lesions there were large and signifi-
Table 2. Compartmentalization of Tissue Water: Distribution and Exclusion Volumes for Three Macromolecules in Normal Intima and Atherosclerotic Lesions

<table>
<thead>
<tr>
<th>Tissue water: mg/100 mg dry tissue</th>
<th>Distribution volume</th>
<th>Exclusion volume</th>
<th>Distribution volume: % of total tissue water</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>LDL</td>
<td>α2-M</td>
<td>Alb</td>
</tr>
<tr>
<td>Normal intima (n = 11)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Gelatinous thickenings (n = 11)</td>
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<tr>
<td>White caps of plaques (n = 6)</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Values are means ± SEM; Alb = albumin.

Significant increases in both distribution and exclusion volumes compared with normal intima (distribution volume: LDL, p = 0.01; α2-M, p = 0.005; albumin, p = 0.002. exclusion volume: LDL, p << 0.001; α2-M, p = 0.01; albumin, p = 0.02; t-test for independent samples). Presumably, increased distribution volume represents increased interstitial space; large spaces between strands of collagen and smooth muscle cells are a characteristic feature of the microscopic appearance of gelatinous lesions. The increase in exclusion volume probably indicates increased hydration of cells and connective tissue elements; for the two larger molecules (LDL and α2-M), but not for albumin, this compartment of the tissue water increases to a slightly greater extent than the interstitial fluid compartment. This can be seen more clearly in Table 3 where the levels in lesions are expressed as percentages of the level in normal intima from the same aorta.

In the transitional lesions, the distribution volume was less than one-half that in the gelatinous lesions (Table 2) and significantly less than the distribution volume in normal intima from the same aorta (Table 3: p = 0.001, t-test for paired samples).

Distribution Volume in Relation to Molecular Mass

In normal intima there is a precisely linear relation between the logarithm of molecular mass and absolute distribution volume, distribution volume as percentage of total tissue water, or distribution volumes of α2-M and LDL expressed as a percentage of the distribution volume of albumin. This latter relation-

Table 3. Distribution and Exclusion Volumes for Macromolecules in Atherosclerotic Lesions Compared with Normal Intima from the Same Aorta

<table>
<thead>
<tr>
<th>Lesion</th>
<th>Distribution volume</th>
<th>Exclusion volume</th>
<th>Distribution volume: % of total tissue water</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>LDL</td>
<td>α2-M</td>
<td>Alb</td>
</tr>
<tr>
<td>Gelatinous thickenings (n = 8)</td>
<td>179 ± 32</td>
<td>171 ± 44</td>
<td>177 ± 46</td>
</tr>
<tr>
<td>Transitional lesions (n = 5)</td>
<td>95 ± 6</td>
<td>61 ± 12</td>
<td>78 ± 18</td>
</tr>
<tr>
<td>White cap of plaque (n = 1)</td>
<td>57 ± 6</td>
<td>23 ± 11</td>
<td>61 ± 18</td>
</tr>
</tbody>
</table>

Values are means ± SEM. Alb = albumin.

*Water content was calculated as mg water/100 mg defatted dry tissue.
The isolation of IF from aortic intima on filter paper inserted into natural cleavage planes enables one to study the environment of the intimal smooth muscle cells without homogenization, application of an electric field, or other manipulation that might disrupt complexes or interactions between plasma macromolecules and components of the connective tissue matrix.¹ Previously we found that, in normal intima in which the smooth muscle cells showed neither focal proliferation nor accumulation of fat, the concentration of LDL in the intimal IF was more than twice the concentration in the patient's own plasma.¹ In atherosclerotic lesions, the concentrations were variable and closely related to morphological characteristics.²

In the present study, the concentration of macromolecules in the IF, their content in adjacent samples of intact tissue, and the total tissue water have been used to estimate the compartmentalization of water in developing proliferative lesions. Only lesions with a low lipid content (based on histological staining and measurement of cholesterol content) were studied, because fat-filled cells appear to destroy LDL and we did not know how to calculate the excluded volume fraction due to lipid.

We concluded previously¹ that there was no evidence of preferential adsorption of LDL on filter paper, which would make its concentration in IF appear artificially high. The LDL concentration in filter papers soaked in serum for 3 hours was 97% of the serum concentration, in papers drained for 10 seconds; it increased to 107% in papers drained for 30 seconds, but some water was probably lost by evaporation. The ratio LDL/α₂-M was the same at 10 and 30 seconds, and about 8% lower than in serum. This probably reflects a larger distribution volume in the paper for the smaller α₂-M molecule rather than preferential adsorption of α₂-M. In experiments in which filter paper was treated with serum, equilibrated for 5 minutes, and then either blotted for 5 seconds or left in contact for 10 minutes with a second paper, concentrations of LDL and α₂-M were decreased in the first paper and increased in the second paper, suggesting that about 5% of the serum water had entered a compartment in the first paper from which macromolecules were excluded.¹

Experiments on the effect of time of insertion in intima on IF collection also suggested that water and macromolecules may be taken up independently. In relatively dry normal intima the weight of IF collected after 5 seconds was 58%, but its concentration of LDL was 155% of the values found after 10 minutes. This suggests that, during the initial 5-second “blot,” the paper absorbed most of the immediately adjacent IF, and during the subsequent 10 minutes, mainly water was absorbed, possibly from the excluded volume compartment. At 10 minutes, the weight of fluid was 98% of the weight collected at 3 hours, but its LDL concentration was only 66% of the 3-hour level. Thus, at 3 hours, LDL concentration was 2% less than at 5 seconds, but nearly twice the weight of fluid had been collected, presumably because there had been time for the IF to re-equilibrate with the surrounding tissue. All samples of IF were collected on papers inserted for 3 hours, and the mass of fluid collected, expressed as mg fluid/100 mg paper, was virtually the same in normal intima and all types of lesion irrespective of their total water.
content (Table 1), suggesting that the IF was fully equilibrated.

It has frequently been postulated that LDL in intima forms complexes with glycosaminoglycans. If LDL were removed from these complexes by electrophoresis of the tissue, this would invalidate the calculations of distribution and exclusion volumes for LDL. However, direct comparison of IF and adjacent intact intima provided no evidence of reversible interaction between LDL and components of the connective tissue matrix.1,2 The ratios of LDL to both α2-M and albumin were consistently lower in tissue than in IF, presumably reflecting their larger distribution volumes, and LDL lay in its expected position in plots of distribution or exclusion volume in relation to molecular mass (Figure 2).

Another possible source of error in this study is redistribution of tissue water following cell death. Theoretically, because of the high colloid osmotic pressure of the intracellular proteins, failure of the ATP-dependent sodium pump will allow sodium and water to flow into the cell.7 In experiments with tissue slices, the water content of kidney cortex incubated at 0°C increased by 30%,7 but in myocardium with irreversible ischemic damage, tissue water increased by only 14%.8 From freeze/thaw experiments to disrupt cells, Harrison and Massaro9 showed that about 20% of tissue water in pig aortic media was intracellular. Presumably water enters the cells from the distributed volume compartment, so that distribution volumes might be underestimated and exclusion volumes overestimated by about 6%. However, it is not clear that cell swelling occurs in situ; following coronary artery occlusion, irreversibly damaged myocardium did not show cell swelling until it was reperfused, or sliced and suspended in Krebs-Ringers phosphate solution.9,10 There is no evidence that arterial smooth muscle cells become permeable to large macromolecules in the postmortem period. By immunofluorescent microscopy, Walton and Williamson11 showed, in autopsy material, an extracellular location of LDL and fibrinogen, and none was located within cells.

In normal intima, about half the total tissue water was in a compartment that was accessible to the large plasma macromolecules LDL (relative molecular mass, M, = 2.4 × 10^6) and α2-M (M, 720,000); an additional 10% of the tissue water was accessible to the smaller albumin molecule (M, 68,000). In the proliferative gelatinous lesions, total tissue water increased by 80% and the distribution volume for the large molecules by about 75%; this is compatible with the appearance of the lesions, which have characteristic large spaces between the bundles of collagen fibers and smooth muscle cells.3,12,13 However, the compartment from which LDL and α2-M were excluded increased by 95%, and the structural basis for this is less easy to define.

Histological sections of the gelatinous lesions were scored for cellularity, the characteristics of the collagen, and the amount of space between the collagen bundles, then grouped into those with the highest or lowest distribution volumes and highest or lowest exclusion volumes. Cellularity was not different in the four groups; the only consistent finding was of wide spaces in the group with the highest distribution volumes.

Acellular areas, which may result from cell necrosis, occur not only in the deepest layer of proliferative lesions, but also at depths from the luminal surface at which they should, theoretically, receive an adequate supply of oxygen,14 and the causes of this patchy absence of cells is not clear. DiBona and Powell15 showed that cell swelling and hydration led to cell necrosis in experimental heart infarction, and this suggested that the overhydration of tissue elements, indicated by a large exclusion volume, might have particular pathological significance. However, there was no specific association between a large exclusion volume and the occurrence of acellular areas in either gelatinous or transitional lesions. Translation of biochemical measurements into morphological features presents serious difficulties; Nir and Pfeffer16 attempted to measure the ratio of IF volume to dispersed cellular volume in dog carotid arteries from transmission electron micrographs and concluded that there was about 18% IF, whereas all measurements with tracer molecules show that it is approximately 40% (reviewed in reference 9). In pig aortic media, Harrison and Massaro9 showed that the distribution volume for inulin was about 50% of the total tissue water. About 20% of the water was intracellular, and inulin was excluded from the remaining 30% of water, which was presumably associated with elastic laminae, collagen fibers, and GAG.9

Studies of IF volume and exclusion of macromolecules in the interstitium of various tissues have recently been reviewed by Aukland and Nicolaysen.17 In most studies, exclusion was calculated from the difference in distribution volume of the macromolecule and a small extracellular space marker such as sucrose; there were marked differences in different tissues both in absolute exclusion, and in relative exclusion in relation to molecular mass.18 Both GAG and collagen exclude macromolecules in vitro; the volume excluded by GAG increases with increasing molecular mass, but collagen seems to exclude almost the same volume for all macromolecules with a molecular mass greater than 50,000 to 100,000.17 Exclusion of high molecular weight dextrans from umbilical cord also appeared to be mainly dependent on collagen, because little change was produced by incubation of the tissue with hyaluronidase, and the collagen fibers seemed to admit serum proteins into their interfibrillar space.17,19

In normal intima and gelatinous lesions, there was a virtually linear relation between log_{10} M and exclusion volume, but in transitional lesions and white fibrous caps, there was a marked change, with disproportional exclusion of the larger molecules (Figure 2). The physicochemical basis of this is not clear.
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If the interstitium behaves in the same way as GAG and collagen in suspension in vitro, it implies that exclusion of macromolecules in normal intima and gelatinous lesions is mainly due to GAG, whereas in more advanced lesions it is mainly due to collagen. Collagen comprises about 25% of the total dry weight of normal intima, compared with 2% GAG. In the gelatinous thickenings, collagen increases by about 20% compared with a decrease of 10% in GAG; since the absolute amount of water per 100 mg of dry tissue in the excluded fraction increased by 50% to 100% compared with normal intima, there must be a decrease in the effective concentrations of both GAG and collagen. By contrast, in the white fibrous plaques in which 40% to 60% of the dry tissue was collagen and the total water content was about 60% of normal intima, there must be a very large increase in effective concentration of collagen.

A macromolecule entering the interstitium may be concentrated in the IF because of its exclusion from part of the tissue water.17,18 This could make only a small contribution to the high concentrations of LDL found in IF in normal intima where the distribution volume for LDL was 80% of the distribution volume of albumin, whereas, relative to plasma, the concentration of LDL was four times the concentration of albumin.

In normal intima, the concentration of LDL in the IF fluid is more than twice the concentration in the patient’s own plasma.1 This strongly suggests that LDL is not intrinsically atherogenic, and that local endothelial damage is unlikely to allow more LDL to enter the intima against a twofold concentration gradient. The gelatinous lesions contained large amounts of IF, but in a third of the lesions the concentration of macromolecules was not significantly greater than in normal intima.2 Thus, proliferation was not necessarily associated with an increase in LDL concentration. In gelatinous and the presumably more advanced transitional lesions, there was an inverse relationship between distribution volume and concentration in IF. Between the gelatinous lesion with the largest and the transitional lesion with the smallest distribution volumes, there was an 11-fold difference. The transitional lesion with the smallest distribution volume also had the highest concentrations of macromolecules in the IF; the concentration of LDL was 11 times, and $\alpha_2$-M six times, the concentrations in the patients’ plasma (absolute concentration of LDL 4200 mg/100 ml). Presumably, other large macromolecules, including fibrinogen, are concentrated to an equivalent extent, and this may be one mechanism involved in their precipitation in developing fibrous plaques.

This study does not help us to understand what initiates focal proliferation of smooth muscle cells and collagen. It does, however, suggest that the connective tissue matrix in gelatinous lesions must be in a highly abnormal physicochemical state. Redistribution of tissue water, which appears to shift out of the IF compartment, may lead to extraordinarily high concentrations of large macromolecules in localized areas of the IF, thus increasing the probability of their extracellular deposition.

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Index Terms: atherosclerotic lesions • interstitial fluid • low density lipoprotein • $\alpha_2$-macroglobulin • albumin • distribution volume • exclusion volume • tissue hydration
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