Atherosclerosis and Molecular Pathology: Mechanisms of Cholesteryl Ester Accumulation in Foam Cells and Extracellular Space of Atherosclerotic Lesions

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This article reviews aspects of the molecular pathology of cholesteryl ester accumulation in atherosclerotic lesions. 1. Transcytosis of lipoproteins through cultured endothelial monolayer. 2. Transformation to foam cell from macrophage. 3. Cholesteryl ester deposition in the extracellular space of atherosclerotic lesions. We also discuss the development and use of novel monoclonal antibodies recognizing atherosclerotic lesions and peroxidized lipoproteins prepared from atherosclerotic lesions.

Keywords — atherosclerosis; endothelial cell; foam cell; extracellular space; transcytosis; cholesteryl ester; lipoprotein; peroxidized lipoprotein; monoclonal antibody

Introduction

A characteristic of atherosclerosis is the accumulation of large amounts of lipids, mainly cholesteryl ester, in the arterial wall. Histochemical and ultrastructural observations have shown that these lipids accumulate in both the cytoplasm of foam cells and the extracellular space. It has been suggested that the foam cells originate from macrophages8-10 and/or modified smooth muscle cells.5,9 It has also been suggested that the extracellular lipids originate from circulating lipoproteins penetrating endothelial cells and/or denatured lipoproteins derived from ruptured foam cells.8,10 The lipids deposited in the extracellular space may be endocytosed by macrophages and/or modified smooth muscle cells, resulting in the formation of foam cells.

1. Transport of Macromolecules through Endothelial Cells in Monolayer Culture

1.1. Transcellular Transport of Lipoprotein — The vascular endothelium is believed to act as a selective barrier to the passage of macromolecules between the blood plasma in the vascular lumen and the interstitial fluid in the perivascular spaces. From in situ morphological studies, Simionescu et al.11-15 showed that various tracers, such as ferritin and horseradish peroxidase, pass through the endothelium either via transcytotic vesicles or through intercellular junctions. Low molecular weight macromolecules tend only to pass through intercellular junctions,11,15,16 the transport appears to be regulated to some extent by both the surface charge of the macromolecules14,17-19 and the charge of the cell surface in the microvascular endothelium.13,20

Studies of the transport of low density lipoprotein (LDL) are important for determining the mechanism of cholesteryl ester accumulation in the arterial wall. Extensive studies of the internalization of LDL through receptors have been made on cultured endothelial cells.16,21,22 Recent cytochemical studies have shown that LDL passes through the endothelium in transcytotic vesicles in situ.23,24 However, very little is known about the mechanism, kinetics and requirements of the transcellular transport of LDL across the endothelium. Territo et al.25 developed a relevant in vitro model. They cultured endothelial cells as a monolayer on a polycarbonate filter and examined the effect of monocytes on 125I-LDL transport through these endothelial cells. We20 also developed a new transcellular transport model using cultured endothelial cells prepared from porcine arterial walls by a slight modification of the method of Neichi et al.27 Endothelial cells were cultured in Dulbecco’s modified Eagle’s minimum essential
medium supplemented with 10% fetal calf serum. After 2 to 3 passages, trypsinized cell suspensions ($8.0 \times 10^5$/dish) were seeded on type I collagen gel prepared from guinea pig skin\textsuperscript{28} supported by a dacron sheet (50 and 75 deniers).

Light microscopy showed that endothelial cells cultured in this way were confluent 2—3 d after seeding (Fig. 1). At this time a well-organized, spindle-shaped cell monolayer was observed on the lattice of the dacron sheet. Scanning electron microscopy showed that the surfaces of the cells cultured on the collagen gel were covered with microvilli and that the cells appeared morphologically similar to cells in vivo (Fig. 2). No cellular detachment was found. Transmission electron microscopy showed several tight junctions along the same intercellular space (Fig. 3), which were comparable to those found between endothelial cells in vivo. In the cells there were many vesicles present along the apical plasmalemma and many small vesicles apparently fusing with the plasma membrane in the basal region. Within 2 d, basement membrane-like materials were present beneath the cell monolayer on the collagen gel (Fig. 4).

To confirm the formation of cellular junctions we measured the electrical resistance of the endothelial barrier. The resistance of the endothelial monolayer increased to $5—10 \ \Omega \cdot \text{cm}^2$ after culture for 1 d, reaching a plateau as the monolayer reached confluence. It remained steady for up to 4 d before decreasing as the cells degenerated (Fig. 5). There are fewer intercellular junctions in the arterial endothelium than are found in MDCK epithelial cells,\textsuperscript{29,30} cultured
endothelial cells from microvessels of brain and adrenal cortex. Consistent with this fact, the resistance of the arterial endothelial cell monolayer was less than that of both MDCK cells (approximately 40 Ω·cm²) and adrenal cortex microvascular endothelial cells (approximately 70 Ω·cm²).

Recently, similar in vitro transport systems with cells from different tissues have been developed. Bowman et al. used a cultured brain endothelial monolayer to study the permeation of sucrose, and Furie et al. cultured microvascular endothelial cells from bovine adrenal cortex on amnion membrane.

Rhodamine B labelled LDL (RB-LDL) can be used as the fluorescent probe of transport but it is slightly different from native LDL because its surface is rather acidic. A considerable amount of the RB-LDL was transported at 37 °C, but not at 0 °C (Fig. 6), and on reducing the temperature from 37 °C to 0 °C, transport decreased dramatically. Vasile et al. concluded from their cytochemical studies that LDL

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Fig. 4. Electron Micrograph of Porcine Endothelial Cells Cultured on Collagen Gel for 2 d after Seeding
The cells are resting on basement membrane-like material. There are many plasmalemmal vesicles along the apical side of the plasmalemma (arrows) and numerous small vesicles fusing with the plasma membrane in the basal region (arrowheads). Bar, 0.5 μm × 24000.

Fig. 5. Change of Electrical Resistance of Cultured Endothelial Cells after Their Seeding on Collagen Gel
The net resistance is shown as Ω·cm². Points and bars are the means ± S.E. for the numbers of experiments shown in parentheses.

Fig. 6. Time Course of RB-LDL Transport
RB-LDL (0.2 mg protein/ml) was introduced into the upper compartment, and the RB-LDL transported through the membrane to the lower compartment was monitored: dacron sheet alone (■); dacron sheet with gelated collagen (△); endothelial monolayer on the dacron sheet with gelated collagen at 37 °C (○), at 0 °C (□), and at 37 °C for 2 h then at 0 °C (▲). The temperature was reduced at the time indicated by the arrow.
transport was not temperature-dependent, since they detected no difference in the transport of LDL at 0 °C and 37 °C. However, it is difficult to compare such morphological finding directly with our in vitro data. In other in vitro systems, the transport of proteins such as bovine serum albumin\textsuperscript{33,34} and insulin\textsuperscript{35} through endothelial cells has been found to be temperature-dependent. The transports of thyroglobulin from the inside to the outside of thyroid follicles\textsuperscript{36} and of vesicular stomatitis virus G protein from the apical to basal plasma membrane of MDCK cells\textsuperscript{37} also show temperature-dependence.

In our study\textsuperscript{26} LDL transport was shown to be energy-dependent, since it was inhibited by a combination of 2-deoxyglucose and NaN\textsubscript{3}, inhibitors of ATP generation (Fig. 7). We also
found that LDL transport was saturable. Transport increased with increasing concentrations of LDL, reaching a maximum at 0.4 mg protein/ml (Fig. 8). The maximal transport rate was approximately 1.3 μg protein/cm²/h, suggesting that LDL did not pass through the endothelial monolayer freely and that its transport must be regulated in some way. After transport at 37 °C no degradation products of apoprotein B were detected by SDS-polyacrylamide gel electrophoresis (Fig. 9), suggesting that 125I-LDL was not metabolised during transport.

These results suggest that LDL is transported in transcytotic vesicles by a temperature- and energy-dependent process, but not through cellular junctions nor by endocytosis and exocytosis via a lysosomal system.

1.2. Intercellular Transport of Macromolecules — Macromolecules have been shown to cross the endothelial cell monolayer by a variety of different transcellular transport mechanisms, including vesicular transport (transcytosis) and transport through transcellular channels and/or intercellular spaces (junctural transport). Simionescu et al. have reported that the rate of transport of electron-dense tracers, such as hemoglobin, horseradish peroxidase and ferritin, through the capillary venular junctions is dependent upon their molecular size. Dextran labelled with fluorescein isothiocyanate (FD) appears to be a good tracer with which to study the vascular permeability of various tissues in situ, since FD is available with a wide range of molecular weights and can be measured quantitatively.

The rate of transport of FD through the endothelial monolayer was indeed dependent on the molecular weight of dextran used (Fig. 10). The results were consistent with histological findings in situ. Simionescu et al. have reported some effects of molecular weight on intercellular transport, based on histological evidence and electron dense probes. The rate of transport of hemoglobin (1.9 kDa) was the highest. Only about 50% of the horseradish peroxidase (40 kDa) passed through the open junctions of the venular endothelium of mouse diaphragm. Very little of the hemoglobin (68 kDa) passed through and almost no (<5%) ferritin (450 kDa). These results are consistent with our findings that dextrans of various sizes are transported through a cultured arterial endothelial monolayer at different rates.

We have also examined the effects of certain chemicals on dextran transport through the endothelial monolayer. Treatment of the cells with cholic acid or cholate B, both known to dissociate cytoskeleton, could cause separation of the tight junctions of cultured cells. Both compounds markedly increased the passage of 4 and 70 kDa of FD; 10 μM cholic acid causing 3.2-fold and 7.1-fold increases, and 5μ/ml cholate B causing 6.1-fold and 22-fold increases respectively. Removal of ionized calcium from the medium with ethylenediaminetetraacetic acid (2.5 mM) also induced separation of the junctions, resulting in a 15-fold increase in the transport of the 4 kDa and a 22-fold increase in that of 70 kDa of FD. Our findings are consistent with those of Bowman et al. Destruction of the cellular junctions by these chemicals enhanced the passage of dextran, which indicates...
that dextran is transported through a monolayer via the intercellular spaces. Vasoactive mediators, such as histamine and serotonin, had no effect on FD transport in this system.

In studying the function of the blood brain barrier, Tervo et al. injected dextran of various molecular weights (3—150 kDa), and found that the capillaries of the cerebral cortex were impermeable to all the intravenous tracer substances used. Measurements of transport rates of dextran in vivo, compared with those found in our in vitro system, may help our understanding of the construction and integrity of the cellular junctions of the endothelial monolayer, which seem to vary with the organ from which the cells are derived.

We concluded that LDL is transported in transcytotic vesicles (not through cellular junctions or by endocytosis and exocytosis via a lysosomal system) because its transport was temperature- and energy-dependent and saturable. In contrast, dextran probably passes through the intercellular junctions of the endothelial monolayer since its transport is temperature-independent and non-saturable (Table I).

| Table I. The Comparison between RB-LDL and FD Transport through the Arterial Endothelial Cell Monolayers |
|-----------------------------------------------|-----------------------------------------------|
| **Molecular**                                | **Molecular**                                |
| **weight (kDa)**                             | **weight (kDa)**                             |
| **FD**                                       | **RB-LDL**                                   |
| 4                                            | 0 °C                                         | 1000—1500                                  | 37 °C                                         |
| 10                                           | 1.8                                          | 0.0                                         | 0.5—0.7                                      |
| Permeation rate (%/h)                        | Temperature-independent                       | Temperature-dependent                       |
| 20                                           | 1.5                                          | 0.6                                         | Unsaturable                                   |
| 70                                           | 0.6                                          | 0.4                                         | Saturable                                     |
| 150                                          |                                              |                                              |                                              |

1.3. Effect of PG12 on Intercellular Transport of Macromolecules through an Arterial Endothelial Monolayer —— Once endothelial cells are injured, the accumulation of lipoproteins such as LDL increase. This appears to be a primary event in atherosclerosis. The endothelial cell acts as a protector against thrombosis. Prostacyclin (PG12), mainly produced in the endothelial cells, is one of the most potent known inhibitors of platelet aggregation and is probably involved in the physiological mechanism preventing thrombosis on the endothelium. In pathological conditions, such as hyperlipidemia and atherosclerosis, where the en-

![Fig. 11. Structures of PG12 and Stable PG12](image)

![Fig. 12. Dose-Response Curves of Prostaglandins](image)

The inhibitory effects (%) on FD transport in 2 h of various concentrations of PG12 (○), stable PG12 (●) and PGE1 (△) are shown.
cholesterol ester may be damaged, there is an associated decrease in production of PGI₂. Decreased levels of PGI₂ may be one of the most important events in injury.

In our studies we have found PGI₂ and isocarbocyanin (stable PGI₂) (Fig. 11) at 3 × 10⁻⁹ M caused 21% inhibition of transport of FD through an endothelial cell monolayer (Fig. 12). The inhibitory effect of the stable PGI₂ persisted for at least 4 h and that of PGI₂ persisted for 2 h (Fig. 13). PGE₁ was the least inhibitory. Our finding that the inhibitory effect PGI₂ was observed for at least 2 h (Fig. 13), although the half-life of native PGI₂ is only a few minutes, suggesting that a secondary mediator was involved in the inhibition.

The inhibitory effect of prostaglandins could be due to increased production of the secondary mediator cAMP. cAMP content was found to increase in the presence of concentrations of PGI₂, which were sufficient to inhibit dextran transport (Table II). To confirm the involvement of cAMP in this system, we tested the effects of some reagents which modulate production of cAMP. In the presence of 3-isobutyl-methylxanthine (IBMX), an inhibitor of cAMP phosphodiesterase, stable PGI₂ increased the cAMP content from 3.3 to 17.1 pmol/10⁶ cells.

![Graph showing time courses of FD transport in the presence of prostaglandins](image)

**Table II.** Cyclic AMP Contents in the Presence of Reagents that Affects Its Production

<table>
<thead>
<tr>
<th></th>
<th>None</th>
<th>DDA</th>
<th>None</th>
<th>DDA</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>1.8 ± 0.2</td>
<td>1.3 ± 0.2</td>
<td>3.8 ± 0.6</td>
<td>1.0 ± 0.3</td>
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<tr>
<td>PGI₂</td>
<td>3.2 ± 0.6</td>
<td>1.3 ± 0.2</td>
<td>13.9 ± 0.3</td>
<td>3.0 ± 0.4</td>
</tr>
<tr>
<td>Stable PGI₂</td>
<td>3.3 ± 0.5</td>
<td>1.3 ± 0.1</td>
<td>17.1 ± 0.5</td>
<td>3.0 ± 0.2</td>
</tr>
<tr>
<td>PGE₁</td>
<td>1.6 ± 0.3</td>
<td>1.2 ± 0.2</td>
<td>5.9 ± 0.7</td>
<td>1.9 ± 0.3</td>
</tr>
</tbody>
</table>

**Table III.** FD Transport in the Presence of Reagents that Affect the cAMP Content of Cells

<table>
<thead>
<tr>
<th>FD (% inhibition)</th>
<th>FD (% inhibition)</th>
<th>FD (% inhibition)</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>Stable PGI₂</td>
<td>PGI₂</td>
</tr>
<tr>
<td>76.3 ± 10.7</td>
<td>44.1 ± 5.9 (42.2)</td>
<td>42.5 ± 7.5 (44.3)</td>
</tr>
<tr>
<td>dbcAMP</td>
<td>+ IBMX</td>
<td>+ IBMX</td>
</tr>
<tr>
<td>49.8 ± 7.4 (34.7)</td>
<td>30.4 ± 6.0 (60.2)</td>
<td>23.2 ± 3.2 (69.6)</td>
</tr>
<tr>
<td>IBMX</td>
<td>+ DDA</td>
<td>+ DDA</td>
</tr>
<tr>
<td>56.5 ± 9.6 (26.0)</td>
<td>70.9 ± 6.5 (7.1)</td>
<td>64.3 ± 11.2 (15.7)</td>
</tr>
<tr>
<td>DDA</td>
<td>+ IBMX</td>
<td>+ IBMX</td>
</tr>
<tr>
<td>73.3 ± 13.3 (3.9)</td>
<td>68.2 ± 9.3 (10.6)</td>
<td>78.1 ± 8.6 (0)</td>
</tr>
</tbody>
</table>

![Image showing FD transport in the presence of reagents affecting cAMP content](image)
A connection between cAMP and FD transport was supported by the evidence obtained using forskolin, a direct activator of the catalytic unit of adenylate cyclase.\textsuperscript{61,62} The production of cAMP was stimulated by 50 \(\mu\text{M}\) forskolin from 1.8 to 5.8 pmol/10\(^6\) cells whilst FD transport was suppressed by 41\%. Similar results have been reported for the transport of Evans-blue through endothelial cells from the human umbilical vein in the presence of PGE\(_1\).\textsuperscript{63}

The inhibitory effects of PGI\(_2\) on FD transport may be explained by an increase in tight junctional connections between endothelial cells, since PGI\(_2\) increased the electrical resistance between the apical and basal layer\textsuperscript{32} from 18.4 to 26.6 \(\Omega\cdot\text{cm}^2\). This interpretation is supported by a correlation between electrical resistance and the cAMP content of epithelial cells.\textsuperscript{64} The barrier enhancement may also be associated with increased endothelial apposition and an increase in polymerized actin activated by cAMP-dependent protein kinase.\textsuperscript{65} Cyclic AMP, which increases with PGI\(_2\) treatment, probably acts as a secondary mediator in stimulating production of tight junctions and cytoskeletal assembly.

2. Accumulation of Cholesteryl Ester in Arterial Wall

2.1. Cholesteryl Ester Deposits in Extracellular Matrix — In early lesions lipid accumulates mainly in the cytoplasm of cells, but in more advanced lesions they are found in the extracellular matrix. By thin section electron microscopy, several investigators have suggested that the electron dense particles are correspond to extracellular lipids.\textsuperscript{8,60} However, due to the limitation of the thin section method the fine structure of the extracellular lipids has not been shown, the neutral lipids being extracted during the dehydration procedure. Using a quick-freeze and etching technique, we\textsuperscript{67} have clearly demonstrated the ultrastructural characteristics

Fig. 14. Appearance of the Extracellular Connective Tissue Space of Atherosclerotic Aorta by the Quick-Freeze, Etching Technique

Large amounts of lipids with a vesicular structure are observed among collagen fibers, which show a characteristic repeating pattern (arrowhead). Some of these vesicular structures appear to be covered by a membrane. Arrows indicate fractured faces of vesiculated lipids corresponding to the P-fracture face of biomembrane. These vesicular structures correspond to vesicles rimmed with an unit membrane structure (inset, arrow) among collagen fibers (inset, arrowhead) in thin sections. (\(\times 44000\); inset, \(\times 40000\)).
of the extracellular lipids. They appeared in the form of irregularly-shaped vesicles and some were observed to have a membrane on their surface (Fig. 14). The vesicles and electron-lucent structures among collagen fibers in thin sections (Fig. 14, inset) corresponded to these extracellular lipids. Some extracellular vesicles were aggregated and the clustered vesicles were similar in size to the cells (Fig. 15), which were destined to collapse in the atherosclerotic aorta. Some extracellular vesicles containing lipids could be derived from these disrupted foam cells. At the same time, lipoprotein or denatured lipoprotein transported through the endothelial barrier could be deposited in the extracellular space. 8)

These extracellular lipids appear to be endocytosed and to accumulate in lysosomes, since the appearance of the extracellular lipid was very similar to that of lipid in the multivesicular body of foam cells (Fig. 16). Brown and Goldstein 2) proposed a scavenger system, observing marked accumulation of cholesteryl ester in cultured macrophages using modified low-density lipoprotein. 68) The endocytic activity of foam

Fig. 15. Appearance of Extracellular Space of Atherosclerotic Aorta by the Quick-Freeze Etching Technique

A plate crystal (Cr) and many vesicular structures are observed. In the lower left an aggregation of vesicles is observed (arrowheads). The surface of the aggregate is smooth. Arrow indicate collagen fiber. (×26000).

Fig. 16. Quick-Freeze Replica of the Perinuclear Area of a Foam Cell

An organelle containing various sized granules is seen among onion-like droplets. Part of the membrane of this organelle shows an E-fracture face (ef). The fractured face of some granular contents are smooth and that of others contains membrane particles (arrow). (N), nucleus (×20000)

Fig. 17. Quick-Freeze Replica of Two Adjacent Typical Foam Cells

The cytoplasm is filled with numerous granules with an onion-like lamellar structure. Smaller granules surrounded by a membrane (mem) are seen among these onion-like granules. A few plate crystals (Cr) are also seen. (N), nucleus; (ins), intercellular space. (×13700).
cells was demonstrated by phase contrast cinemicrophotography. The vesicular extracellular lipids observed in our studies may correspond to denatured lipoproteins endocytosed by scavenger cells such as macrophages, modified smooth muscle cells and foam cells, and foam cell transformation may be accelerated.

2.2. Accumulation of Cholesteryl Ester in Foam Cells — In early lesions lipids accumulate mainly in the cytoplasm of cells such as macrophages. Thin section electron microscopy has shown that intracellular lipids are stored in two forms in the cytoplasm of foam cells: in membrane-bound vacuoles, which probably correspond to lysosomes, and in vacuoles without a peripheral membrane. These lipid droplets, both with and without membranes, were also observed in quick-freeze replicas of foam cells which may be derived from macrophages (Fig. 16, 17) and smooth muscle cells. The membrane-free droplets were more numerous than membrane-bound droplets, and appeared to consist of onion-like concentric lamellae. Similar onion-like lamellae have previously been observed using the conventional freeze-fracture method. This lamellar structure suggests that the constituent lipids of the droplets are in an orderly arrangement. This molecular arrangement of lipids is consistent with models proposed by Hata and Insull and Engelmann and Hillman from the anisotropic nature and X-ray diffraction pattern of cholesteryl ester-rich inclusions. Onion-like lamellae were observed in anisotropic cholesteryl ester droplets prepared in vitro. These studies suggest that onion-like droplets consist mainly of cholesteryl ester and correspond to the cholesteryl ester-rich lipid inclusions isolated by Lang and Insull and by us.

More detailed examination of the membrane-free droplets by the thin section and the quick-freeze etching methods showed that these onion-like lipid droplets were surrounded by 10 nm filaments (Fig. 18). The existence of similar filaments has been observed in lipid droplets of cholesterol-loaded macrophages and in triglyceride droplets in adipocytes.

The other type of lipid droplets in foam cells are membrane-bound (Figs. 16, 18) as found in lysosomes. These lipid-laden organelles may correspond to low-density lysosomes, that may be prepared by flotation sucrose density gradient centrifugation. The lipid droplets in lysosomes vary in size.

Fig. 18. Thin Section of the Cytoplasm of a Foam Cell
Lipid droplets are observed as vacuoles with (Ly) and without (L) membranes. Some membrane-free droplets are observed surrounded by 10 nm filaments (arrow). These filaments (arrow) are also seen associated with onion-like droplets in quick-freeze replicas (inset). (× 29000; inset, × 47000).
Fig. 19. Lipid Droplets Prepared by Flotation Sucrose Density Gradient Centrifugation
(A) Scanning electron micrograph. (×17000). (B) Polarized micrograph. (×2500).

In order to examine the lipid composition of droplets in foam cells, these droplets were isolated by flotation sucrose density gradient centrifugation. The particles appeared spherical (1—3 μm in diameter) by scanning electron microscopy (Fig. 19), and anisotropic cross images were observed by polarized microscopy (Fig. 19). These lipid droplets consisted mainly of cholesteryl ester (about 95%). Free cholesterol, phospholipids and triglycerides accounted for less than 5%. The fatty acid composition was investigated using gas liquid chromatography.

Fig. 20. Cultured Foam Cells Prepared from Atheromatous Aorta by Enzyme Digestion (×600)
(A) Phase contrast micrograph. Numerous granules are seen in two cells. (B) Polarized micrograph. One cell contains many anisotropic lipid droplets and the other few.
Fig. 21. Cholesteryl Ester Liquid Crystals in Macrophages

(A) Mouse peritoneal macrophages were plated in culture dishes and then incubated with cholesteryl oleate liquid crystals (50 μg/ml) in the presence of esterastin (5 μM) for 24 h. The cells were fixed and processed for electron microscopy, ×9500 (inset: high magnification views. (a) ×24000, (b) ×15700, (c) ×162000). Arrows indicate the boundaries of lipids. (Lp1) lipid surrounded by a limiting membrane. (Lp2) lipid droplet without a limiting membrane. (Ly) lysosomal components. (B) After washing medium from the cells, the cells were further incubated in fresh medium in the presence of 5 μM esterastin for 48 h. ×14500 (inset; high magnification view, ×65000).
Cholesteryl olate accounted for the greatest proportion (53%), whilst both cholesteryl linoleate and linolenate were present in only small amounts. Anisotropic liquid crystals were also observed when the foam cells are cultured *in vitro* (Fig. 20A,B).69

Goldstein *et al.*68,69 obtained suggestive evidence that foam cells were derived from macrophages. When macrophages were incubated with acetylated low density lipoprotein (acetyl-LDL), the acetyl-LDL was internalized through endocytosis and then delivered to lysosomes. Cholesteryl esters in the lipoprotein were hydrolyzed in lysosomes, and free cholesterol was liberated. According to the hypothesis of Brown *et al.*68 the free cholesterol is reesterified by a microsomal acyl-CoA: cholesterol acyltransferase. The resulting cholesteryl esters accumulate as cytoplasmic lipid droplets. Similar results were obtained using malondialdehyde LDL,86,87 dextran sulfate LDL complex,88 LDL proteoglycan complex,89,90 endothelial cell-modified LDL,91 cholesteryl ester-protein complex,92 and β-VLDL,93

On the other hand, in highly lipid-laden cells, elements of the endoplasmic reticulum which involves in reesterification of cholesterol are rarely found (Fig. 17).67 An alternative explanation for the accumulation of membrane-free droplets in these highly lipid-laden foam cells is that lipid inclusion bodies accumulate, not *via* lysosomes, but are transferred to the cytoplasm from phago-lysosome with partial hydrolysis of cholesteryl esters. This explanation is analogous

![Fig. 22. Effect of Esterasin on the Hydrolysis of Cholesteryl Ester Liquid Crystals](image)

Macrophages were incubated with liquid crystals (4.8 μg/ml, 1,400 cpm/nmol of [3H]cholesteryl olate) for the indicated times in the presence of 0 μM (○), 1 μM (□) 29, 5 μM (▲) or 25 μM (■) esterasin. The amount of free cholesterol is indicated as a percentage of the total cholesterol in the cells.

![Fig. 23. Working Hypothesis for Cholesteryl Ester Accumulation in Macrophages](image)

Ly, lysosome; FC, free cholesterol; N, nucleus; ER, endoplasmic reticulum; PG, phagolysosome; ED, endosome.
to the pathway involving vitamin A-rich lipid droplets proposed by Wake. This possibility may be examined by investigating the transition from membrane-bound to membrane-free droplets. After 24 h incubation with cholesteryl oleate liquid crystals prepared in vitro, most lipid inclusion bodies in the cytoplasm were surrounded by a limiting membrane, suggesting that they correspond to phago-lysosomes (Fig. 21A). Esterastin, a lipase inhibitor, was used in this experiment to inhibit the hydrolysis of cholesteryl esters. We found that the $K_i$ value of esterastin for purified acid lipase was approximately 90 nM. After incubation with 5 $\mu M$ esterastin for 12 h, the levels of hydrolysis and efflux of cholesterol were less than 5% and 25% of the control levels, respectively (Fig. 22). Even the hydrolysis of cholesteryl esters was suppressed to a great extent: no lipid inclusion bodies surrounded by a limiting membrane could be observed in the cytoplasm (Fig. 21B). Our results suggest that lipid droplets are transferred from phagolysosomes to the cytoplasm via the “vesicular pathway” (path II in Fig. 23).

Most lipid droplets without a limiting membrane appear to comprise a relatively electron-dense rim surrounding an electron-lucent core (Fig. 21B, Lp2). These features were also observed frequently in lipid-laden cells in atherosclerotic lesions and in in vitro cells with lipid inclusion bodies. This suggests that the properties of lipids in lipid inclusion bodies without a limiting membrane may alter when they are located in the cytoplasm. One explanation for this observation is that a compositional change, in terms of double bonds of fatty acids, had taken place and that these are more likely to be oxidized by osmic acid. It is also possible that the phospholipids of lysosomal membrane have dissolved in the lipid inclusion bodies and thus the lysosomal limiting membrane has disappeared. The lipid droplets with an electron-dense rim in cultured cells might arise through the same mechanism as in an atherosclerotic lesions in vivo. These results suggested a new “vesicular pathway” for the accumulation of cholesteryl ester lipid inclusion bodies without a limiting membrane in the cytoplasm. They also may lead to a better understanding of how macrophages change into lipid-laden foam cells in atherosclerotic lesions, since foam cells may be formed after endocytosis of anisotropic lipid droplets derived from ruptured foam cells.

With regard to the mechanism of cholesteryl ester accumulation in foam cells, we found that acid cholesteryl esterase activity was reduced in low-density lysosomes, which may contain a large amount of cholesteryl ester, and that marked accumulation of cholesteryl ester occurred in this organelle. To investigate the reason for reduced acid cholesteryl esterase activity, we purified lysosomal acid cholesteryl esterase from rabbit liver (21000-fold) to a form which appeared to be a single protein by SDS polyacrylamide gel electrophoresis (Fig. 24). We also isolated low-density lysosomal membranes from atheromatous aorta (Figs. 25, 26). The lipid composition of these mem-
Fig. 25. Preparation of Low-density Lysosomal Membranes

The low-density lysosomal fraction was subfractionated by linear sucrose density gradient centrifugation. Low-density lysosomal membranes were recovered in the peak B fraction, in which phospholipids, free cholesterol, and N-acetyl-β-glucosaminidase (lysosomal marker enzyme) were detected. Cholesteryl ester-rich lipid droplets were recovered in the peak A fraction. The shaded area represents the applied layer of sample.

Fig. 26. Electron Micrograph of (A) the Low-density Lysosomal Membranes Recovered in the Peak B Fraction in Fig. 25 (×60000) and (B) Atheromatous Aorta from Which the Membranes Were Prepared (L₁), lipid-laden lysosome; (L₂), lysosome containing myelin-like material. (×25000).
branes was abnormal in the following respects. 1. The molar ratio of free cholesterol to phospholipid was very high. 2. There was a high content of sphingomyelin with a very low amount of phosphatidylethanolamine. 3. The phosphatidylcholine had a low proportion of polyunsaturated fatty acids. These changes in lipid composition of lysosomal membrane should reduce the acid cholesteryl esterase activity for the following reasons. 1. The activating effect of sphingomyelin on purified acid cholesteryl esters was much lower than that of phosphatidylethanolamine. 2. The activating effect of phosphatidylcholine was correlated with the degree of unsaturation of its fatty acid.

3. Monoclonal Antibodies

3.1. Monoclonal Antibodies Recognizing Lipid-Laden Cells and Extracellular Regions with Lipid-Deposits in Atherosclerotic Aorta — In order to study the mechanisms of formation and disruption of foam cells, we prepared novel monoclonal antibodies against atherosclerotic lesions using a delipidated crude homogenate of atherosclerotic aorta of Watanabe-heritable hyperlipidemic (WHHL) rabbits as a complex mixture of immunogens. The monoclonal antibodies were prepared by the hybridoma technique established by Köhler and Milstein. By screening the antibodies histochemically we were able to obtain for the first time monoclonal antibodies which specifically recognized lipid-laden cells (FCR1a/201F, FCR1b/904B) and the extracellular matrix with lipid-deposits (EMR1a/212D) in atherosclerotic lesions.

The monoclonal antibodies of FCR1a/201F and FCR1b/904B specifically stained cytoplasmic faces of lipid-laden cells, but did not stain any arterial cells without lipid (Fig. 27). The staining was not reduced when frozen sections were soaked in acetone or when paraffin sections were delipidated during the dehydration procedure (Fig. 28), suggesting that the epitope against these antibodies is not lipid. Two types of antibody-positive cells, round and elongated, were recognized. The round lipid-laden cells, distributed beneath the endothelium and recognized by these antibodies, may originate from macrophages, since both their shape and the density of their cytoplasm were similar to those of macrophages. The other lipid-laden cells stained by these antibodies were elongated and extended from the internal elastic lamina toward the lumen (Fig. 28). Recently Watanabe et al. found that monoclonal antibody against macrophages

Fig. 27. Correlation of Immunofluorescence of FCR1a/201F Antibody Staining and Lipid-deposits
(A) Both intracellular (F) and extracellular (E) lipid-deposits in atherosclerotic lesions are stained with oil-red O. (Oil-red O and haematoxylin staining, ×300). (B) Only intracellular lipid-deposits show immunofluorescence with FCR1a/201F antibody. (Indirect immunofluorescent staining of the adjacent to that for (A), ×300).
Fig. 28. Staining with FCR1b/904B Antibody of Foam Cells Extending from the Internal Elastic Lamina toward the Lumen
(A) Assembled foam cells and intimal smooth muscle cells extend from the internal elastic lamina toward the lumen. On the left, there is a necrotic area containing needle like crystals of cholesterol. (Haematoxylin eosin staining, ×150).
(B) Immunofluorescence on staining with FCR1b/904B antibody is restricted to the cytoplasm of foam cells. Endothelial cells, smooth muscle cells and the necrotic area are not stained. (Indirect immunofluorescent staining of the adjacent section to that for (A), ×150).

did not stain elongated lipid-laden cells, although it was active against round lipid-laden cells. This suggests that these elongated cells are unlikely to be derived from macrophages, although they may originate from smooth muscle cells. The existence of foam cells of smooth muscle cell origin has also been suggested by many other workers.

Fig. 29. Correlation of EMR1a/212D Antibody Staining and Lipid-Deposits
(A) Two major areas of lipid-deposits are observed in atherosclerotic lesions by oil-red O staining. Large droplets stained with oil-red O in contact with nuclei (F) are lipids in the cytoplasm of foam cells and small scattered droplets near the elastic internal lamina (E) are lipid-deposits in the extracellular matrix. (Oil red O and haematoxylin staining, ×150).
(B) Immunofluorescence on EMR1a/212D antibody staining is restricted to extracellular regions with lipid-deposits. (Indirect immunofluorescent staining of the adjacent section to that for (A), ×150).
FCR1a/201F and FCR1b/904B seem to stain two types of cells of different origins, indicating that they do not stain specific cells, but cells with cytoplasmic accumulations of lipid. Thus these antibodies may recognize some materials induced as a result of intracellular lipid accumulation, regardless of the species of lipid-laden cells.

The antibody of clone FCR1a/212D bound specifically to intimal extracellular regions containing lipid-deposits (Fig. 29) although it is uncertain whether it stained only the intima in advanced atheromas penetrating into the media. It also stained tissue sections that had been delipidated, suggesting that its antigen in the extracellular matrix is not lipid. The antigenic material must be a protein or glycoprotein located in the extracellular matrix, since the EMR1a/212D antibody recognized 3 bands (Mw 40—70 kDa) on immunoblots of a homogenate of atherosclerotic aorta separated by SDS-polyacrylamide gel elec-

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Fig. 30. Effect of Neuraminidase on the Antigenic Activity of Tissue Section of Atherosclerotic Aorta
(A) Immunofluorescence on EMR1a/212D monoclonal antibody staining is observed in the extracellular matrix of atherosclerotic lesions. (Indirect immunofluorescence staining; ×200). (B) After the incubation with neuraminidase (10 mU/ml) at 37 °C for 24 h immunofluorescence on the section adjacent to that for (A) disappeared. (Indirect immunofluorescence staining; ×200).
trophoresis. The purified antigenic material is a glycoprotein with a molecular weight of 66 kDa containing a large amount of sialic acid, and the epitope of the antigenic material contains sialic acid as a major element (Table IV, Fig. 30). Since sialic acid is widely distributed in glycoproteins on the surface of normal animal cells, it seems unlikely that sialic acid should be the epitope of pathogenic tissues such as atherosclerotic lesions. However, Nores et al. 105 showed that GM3 ganglioside, detected by mouse monoclonal antibody against melanoma cells, is the specific site of the surface antigen of melanoma, even though GM3 is widely distributed on animal cells. They concluded that the reactivity of the antibody depends either on the density of GM3 exposed at the cell surface or on a conformational change in a structural protein caused by GM3. They postulated that a high concentration of GM3 in a membrane may induce a novel conformational change which is recognized by the antibody. Therefore, the epitope of our antigenic material may be connected with the configuration of a glycoprotein.

Fig. 31. Distribution of Antigenic Material in WHHL Rabbit Serum

WHHL rabbit serum was adjusted to a density of 1.21 g/ml with NaBr and centrifuged at 25000 rpm for 24 h at 18 °C on a stepwise gradient of NaBr. Solid line, cholesterol; dashed line, density; shaded line, antigenic activity.

Our antigenic material is probably not VLDL, LDL, and apolipoprotein B (apo B) itself, and may not even be associated with VLDL and

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Fig. 32. SDS-PAGE and Western Blot Analysis of Serum and Atherosclerotic Aorta of WHHL Rabbits

WHHL rabbit serum and homogenate of atherosclerotic aorta were separated by electrophoresis in 10 % SDS polyacrylamide gels and stained with coomassie brilliant blue (SDS-PAGE). Nitrocellulose sheets transferred from the gels were stained by EMR1a/212D monoclonal antibody (Western blot). Lane MW, molecular weight standards; lane WA, homogenate of atherosclerotic aorta of WHHL rabbit; lane WS, WHHL rabbit serum.
LDL. Evidence of this comes from the fact that the antigenic activity was detected at a higher density than VLDL and LDL on the NaBr density-gradient centrifugation of serum (Fig. 31) and the antigenic material migrated more slowly than VLDL and LDL on agarose gel electrophoresis. Furthermore, the antibody did not react with the band of large apo B molecule SDS-PAGE by Western blot analysis (Fig. 32). Antigenic material did not react with anti-rabbit apo B antiserum and monoclonal antibody. A major band was found on the Western blots at 66 kDa. Although albumin is a major protein with a molecular weight of 66 kDa, it is not the antigenic material since the antigenic material is a glycoprotein containing sialic acid, but albumin does not contain sialic acid. Additionally, antigenic material migrated more slowly than albumin on agarose gel electrophoresis, and the amino acid composition of the antigenic material was different from that of albumin. Furthermore, the antigenic material did not cross-react with anti-rabbit albumin antiserum. These findings suggest that the antigenic material is not the albumin. The antigenic material is also not HDL since the it migrated more slowly than HDL on agarose gel electrophoresis. We cannot ignore the possibility, however, that the antigenic material may associate with HDL in vivo since the antigenic activity was recovered at the same density as the HDL fraction on NaBr density-gradient centrifugation (Fig. 31). Additionally, a fresh epitope was expressed by the degradation of these macromolecules.

Our results from SDS-PAGE and Western blot analyses and from neuraminidase treatment suggest that the antigenic materials purified from serum and atherosclerotic aorta are similar. Possible antigenic materials which have been shown to exist in the extracellular matrix with lipid deposits are glycosaminoglycans (GAGs), proteoglycans, fibrin, fibrinogen, and some of the glycoproteins. Increase in sulfated GAGs such as chondroitin sulfate, and dermatan sulfate in the aorta showed a positive correlation. Dermatan sulfate in the aorta also correlated positively with increased accumulation of lipids.\textsuperscript{106,107} It has been proposed that GAGs can form complexes with LDL.\textsuperscript{108–112} Increase in proteoglycans such as chondroitin sulfate and dermatan sulfate, proteoglycans also coincided with an increase in lipids.\textsuperscript{113,114} Chondroitin sulfate proteoglycan was detected by anti-chondroitin sulfate proteoglycan monoclonal antibody in thickened intima.\textsuperscript{115} Camejo and colleagues\textsuperscript{116,117} found that arterial chondroitin sulfate proteoglycan exhibits a marked affinity for LDL. Other investigators have also suggested that proteoglycans form complexes with LDL.\textsuperscript{118–120} Fibrin is a major component of lipid-rich areas of atherosclerotic plaque. Smith and colleagues\textsuperscript{121–123} postulated that fibrinogen may be converted to fibrin within lesions since fibrin that has accumulated within the intima seems to bind LDL. Sadoshima and Tanaka\textsuperscript{124} reported that deposition of fibrinogen was associated with deposition of LDL. In addition to these, other uncharacterised extracellular glycoproteins believed to be associated with lipids have been extracted from the aorta.\textsuperscript{124–128} Some glycoproteins found in plasma have also been detected in the atherosclerotic aorta.\textsuperscript{129–131} None of these materials, however, can represent our antigenic macromolecule. EMR1a/212D did not cross-react with fibrinogen as shown by Western blot analysis, although the migration profile of fibrinogen on SDS-PAGE was similar to that of aortic antigenic material. The molecular weight, amino acid composition, and carbohydrate content of our antigenic material differed from those of the above materials. The epitope of the antigen consists at least of sialic acid. We propose, therefore, that the antigenic material recognized by EMR1a/212D may be a glycoprotein hitherto undescribed in the atherosclerotic aorta.

3.2. Monoclonal DLR1a/104G Antibody Recognizing Peroxidized Lipoproteins in Atherosclerotic Lesions — It is thought that peroxidized lipoproteins, one of the modified classes of lipoproteins, are involved in the development of atherosclerosis. However, as yet it is unclear which peroxidized lipoproteins exist \textit{in vivo} and how these are involved in the pathogenesis. We have demonstrated the presence of peroxidized lipids, such as 13-hydroxyoctadecadienoic acid, in atherosclerotic aortas of WHHL rabbits.\textsuperscript{132} Harland \textit{et al.}\textsuperscript{133,134} have also reported similar peroxidized fatty acids in
human atherosclerotic aortas. It has been proposed that the peroxidation of LDL may occur through the interaction of LDL with endothelial cells,\textsuperscript{135,136} macrophages\textsuperscript{137,138} or smooth muscle cells.\textsuperscript{139} Peroxidized lipids may accumulate in extracellular lipids which are partly derived from disrupted foam cells.\textsuperscript{67}

In order to examine the involvement of peroxided lipoproteins in atherogenesis, attempts were made to prepare a monoclonal antibody which recognized lipoproteins through immunization with the float-up fraction prepared from atherosclerotic aortas. We obtained a novel monoclonal antibody (DLR1a/104G)\textsuperscript{140} which is different in specificity from those prepared, by Gonen et al.\textsuperscript{141} using MDA-LDL as immunogen.

A study was undertaken to prepare a monoclonal antibody recognizes peroxidized lipoprotein in order to demonstrate the existence of peroxidized lipoproteins in atherosclerotic aorta. ELISA and immunoblotting techniques demonstrated that the DLR1a/104G monoclonal

![Graph](image)

Fig. 33. Reactivity of the Monoclonal DLR1a/104G Antibody with Modified and Native LDLS as Determined by ELISA

Microtiter plates were coated with modified and native LDLS (0.4—200 µg protein/ml). Peroxidized LDL (○), MDA-LDL (▼), acetyl-LDL (△) and native LDL (□).

![Immunoblot](image)

Fig. 34. Reactivity of the Monoclonal DLR1a/104G Antibody with Modified and Native LDLS as determined by Immunoblotting

(A) Modified and native LDLS (2 µg total cholesterol equiv.) were submitted to 1% agarose gel electrophoresis. Proteins on the gel were stained with coomassie brilliant blue. (B) After electrophoresis, the proteins were transported to nitrocellulose and then immunostained. Lane 1, peroxidized LDL; lane 2, MDA-LDL; lane 3, acetyl-LDL and lane 4, native LDL.
Fig. 35. Antigenic Activity of Peroxidized LDL Extracted with Hexane-Isopropanol

Plates were coated with peroxidized LDL (0.2—27 μg protein/ml). Lipids of peroxidized LDL in the wells were extracted with hexane-isopropanol (3:2, v/v) at 4 °C overnight. Antigenic activity was measured by ELISA (●). As a control, antigenic activity of peroxidized LDL without organic solvent treatment was also determined (○).

antibody reacts with peroxidized lipoproteins (Fig. 33). The antigenic determinant may be associated with peroxidized LDL particles, since the immunostained band recognized by the DLR1a/104G antibody migrated together with that of protein after peroxidation with CuSO4 (Fig. 34). The antigenicity of peroxidized LDL may be associated with a substance in the pro-

Fig. 36. SDS-Polyacrylamide Gel Electrophoresis and Immunoblotting of Peroxidized and Native LDLs

Peroxidized LDL and native LDL (10 μg total cholesterol eqv.) were submitted to 2 % SDS, 3—15 % polyacrylamide gradient slab gel electrophoresis. (A) The proteins on the gel were stained with coomassie brilliant blue, and (B) the proteins transferred to nitrocellulose were determined by immunoblotting. Lane 1, peroxidized LDL; lane 2, native LDL.

*Protein band appearing after peroxidation of LDL.

Fig. 37. Correlation of the Antigenic Activity and the Fluorescent Substances Formed in the Process of LDL Peroxidation

LDL (50 μg total cholesterol/ml) was incubated with 10 μM CuSO4 at 37 °C (○) or 4 °C (●) in D'PBS (—). After the addition of 1 mM dibutyrylhydroxytoluene, the antigenic activity was determined by ELISA (A) and the fluorescence at $E_{350}/F_{410}$ was measured (B).
tein fraction, since the antigenic activity of peroxidized LDL did not decrease on extraction with hexane-isopropanol (Fig. 35), and the immunostained band coincided on SDS-polyacrylamide gel electrophoresis with that of protein after peroxidation with CuSO₄ (Fig. 36). Furthermore, the antigenic determinant may be related to the fluorescent substance in the protein fraction of peroxidized LDL, as suggested by the correlation of the antigenic activity with fluorescence (Fig. 37). The DLR1a/104G antibody may recognize a modified form of apo B, since the peroxidation of LDL results in formation of a fluorescent substance due to the derivatization of apo B by peroxidized lipids.¹⁴²

Gonen et al.¹⁴¹ recently prepared monoclonal EB-3 antibody using MDA-LDL as an immunogen. They demonstrated that EB-3 antibody reacts strongly with MDA-LDL but not significantly with native LDL or other modified LDLs (acetylated, carbamylated or glycosylated LDLs). However, the reactivity of the antibody with peroxidized LDL was not mentioned. According to their results, the lipoprotein fraction prepared from the media or intima failed to react with the EB-3 antibody. Our results showed that float-up fraction prepared from atherosclerotic aortas did react with the DLR1a/104G antibody. These results support the view that peroxidized lipoproteins, which have an antigenic determinant in common with peroxidized LDL, exist in atherosclerotic lesions.

These results suggest the existence of peroxi-

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**Fig. 38.** Cross-Reactivity of the Monoclonal DLR1a/104G Antibody with the Float-up Fraction Prepared from Atherosclerotic Aortas

The purified DLR1a/104G IgM antibody (0.3 μg protein/ml) was incubated with various concentrations of the float-up fraction, and modified and native LDLs at 4 °C overnight. The mixtures were added to the wells, which had been previously coated with peroxidized LDL (1 μg total cholesterol eqv./ml) followed by blocking. The reactivity of the float-up fraction, and the modified and native LDLs was determined as the inhibition of the reactivity of the antibody with peroxidized LDL by ELISA. Float-up fraction (●), peroxidized LDL (○), MDA-LDL (▽), acetyl-LDL (△) and native LDL (wh).

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**Fig. 39.** Our Working Hypothesis on Accumulation of Cholesteryl Ester in Atherosclerotic Lesions
dized lipoproteins in atherosclerotic lesions (Fig. 38), as judged using monoclonal DLR1a/104G antibody which recognized peroxidized lipoproteins in atherosclerotic aortas as well as peroxidized LDL prepared by CuSO₄-catalyzed peroxidation.

4. Conclusions

Figure 39 shows our working hypothesis of the development of atherosclerosis. Lipoproteins in the blood are transported through the arterial endothelium, are then endocytosed by macrophages and smooth muscle cells to be degraded in the lysosomes. Excess lipoproteins are taken up by scavenger cells such as macrophages. The free cholesterol liberated from the lysosomes after hydrolysis of cholesteryl ester is reesterified by microsomal ACAT enzyme, and accumulate in the cytoplasm as anisotropic lipid droplets (Fig. 23, path I). Some lipid droplets may be transferred directly from the phago-lysosomes to the cytoplasm with partial hydrolysis (Fig. 23, path II). Accumulation of excess lipids within the macrophages results in the transformation of macrophages into foam cells. The overloaded foam cells may rupture and release lipid droplets into the extracellular spaces. Extracellular lipids, which may be harmful to living tissue because of their hydrophobicity, are possibly endocytosed and segregated by scavenger cells such as macrophages and modified smooth muscle cells to maintain a hydrophilic environment. The lipids which escape uptake by scavenger cells accumulate in the extracellular spaces of the arterial wall, and may become peroxidized and cause additional harm to tissues.

This article reviews the morphological and molecular pathological aspects of the mechanism of development of atherosclerosis. Recent advances in biochemical techniques should make it possible to further elucidate more details of the mechanisms.

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