Immunohistochemical Demonstration of Enzymatically Modified Human LDL and Its Colocalization With the Terminal Complement Complex in the Early Atherosclerotic Lesion

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Abstract—Treatment of low density lipoprotein (LDL) with degrading enzymes transforms the molecule to a moiety that is micromorphologically indistinguishable from lipoproteinaceous particles that are present in atherosclerotic plaques, and enzymatically modified LDL (E-LDL), but not oxidized LDL (ox-LDL), spontaneously activates the alternative complement pathway, as do lesion lipoprotein derivatives. Furthermore, because E-LDL is a potent inducer of macrophage foam cell formation, we propose that enzymatic degradation may be the key process that renders LDL atherogenic. In this article, we report the production of two murine monoclonal antibodies recognizing cryptic epitopes in human apolipoprotein B that become exposed after enzymatic attack on LDL. One antibody reacted with LDL after single treatment with trypsin, whereas recognition by the second antibody required combined treatment of LDL with trypsin and cholesterol esterase. In ELISAs, both antibodies reacted with E-LDL produced in vitro and with lesion complement activator derived from human atherosclerotic plaques, but they were unreactive with native LDL or ox-LDL. The antibodies stained E-LDL, but not native LDL or ox-LDL, that had been artificially injected into arterial vessel walls. With the use of these antibodies, we have demonstrated that early human atherosclerotic coronary lesions obtained at autopsy as well as lesions examined in freshly explanted hearts always contain extensive extracellular deposits of E-LDL. Terminal complement complexes, detected with a monoclonal antibody specific for a C5b-9 neoepitope, colocalized with E-LDL within the intima, which is compatible with the proposal that subendothelially deposited LDL is enzymatically transformed to a complement activator at the earliest stages in lesion development. (Arterioscler Thromb Vasc Biol. 1998;18:369-378.)

Key Words: atherosclerosis ■ LDL ■ complement activation ■ enzymatic degradation ■ immunohistochemistry

Why LDL trapped in the subendothelium should trigger events leading to chronic inflammation and arterial wall injury is a major enigma of modern medicine. Oxidation of LDL in vitro renders the molecule potentially atherogenic: ox-LDL stimulates monocyte–endothelial cell interactions,1 induces cytokine synthesis and release from macrophages,2,3 and is an apoptotic stimulus in smooth muscle cells.4,5 Ox-LDL is recognized by macrophage scavenger receptors6,7 and is a potent inducer of foam cell formation.8–11 Flanked by reports that ox-LDL can be immunohistochemically detected in rabbit atherosclerotic lesions,12,13 that antibodies against ox-LDL occur in patients with atherosclerosis,14–17 and that antioxidants reduce the risk of myocardial infarction,18–22 these findings have left little room for doubt that ox-LDL is, indeed, the prime initiator of atherosclerosis.9,22–26 Nevertheless, a number of experimental findings do not entirely concur with this concept. First, biochemical analyses have not confirmed the presence of large amounts of oxidized lipid in lipoproteins derived from human atherosclerotic lesions.27–29 In a recent study, only 0.06% to 0.3% of fatty acids isolated from early human lesions were found to be oxidized. Most of those lesions already contained infiltrating macrophages, so the amount of extracellular primarily oxidized lipid must have been yet lower.29 Second, subendothelially deposited LDL particles fuse to form large lipid droplets at a very early stage in lesion development,30–34 well before cellular infiltration by macrophages, lymphocytes, and smooth muscle cells is observed.34 Ox-LDL does not form fused particles displaying a similar micromorphology in vitro. Third, lipid moieties possessing the capacity to spontaneously activate the alternative complement pathway have been isolated from human atherosclerotic lesions.35 Collectively designated LCA, these lipid droplets ultrastructurally resemble lipoprotein derivatives that had been isolated from atherosclerotic plaques in other laboratories.30–33 The lesion lipids contain relatively large amounts of free cholesterol30,32,35,36–37 whereas the bulk of...
cholesterol in both LDL and ox-LDL is esterified. Deesterification is probably important in generating the complement-activating property of LCA, which is not shared by native LDL or ox-LDL.35 Indeed, atherosclerotic human lesions do contain activated complement,35,36–40 and in animal experiments, C5b-9 deposition occurs at very early stages in lesion development.41

To resolve this paradox, we previously attempted to modify LDL in vitro to generate an entity that would morphologically and functionally resemble lesion lipoproteins more closely than does ox-LDL. By combined treatment with trypsin, cholesteryl esterase, and neuraminidase, LDL (but not HDL or VLDL) could indeed be transformed to a complement-activating moiety with an ultrastructure resembling LCA.42 Moreover, E-LDL was found to be recognized by one or several human macrophage scavenger receptors, potentially inducing foam cell formation. These findings led to the hypothesis that enzymatic alteration rather than oxidation of LDL might represent the missing link between lipoprotein deposition and initiation of atherosclerosis.42

Direct demonstration that subendothelially deposited LDL is enzymatically altered at a very early stage in lesion development would strengthen this hypothesis. We anticipated that enzymatic attack on LDL might expose one or several cryptic epitopes, here referred to as neoepitopes, that could become detectable with mAbs. This hope was fulfilled, and because the novel antibodies could be used to stain paraffin sections, we were able to screen a bank of preserved material and to examine a large number of well-defined early human lesions. Control experiments showed that the antibodies did not stain native LDL or ox-LDL. In contrast, the mAbs reacted with E-LDL as well as with LCA particles isolated from human atherosclerotic lesions that contained no detectable ox-LDL. With the use of these antibodies, it became possible to directly demonstrate extensive extracellular depositions of E-LDL in the early human atherosclerotic lesion. C5b-9 deposits colocalized with altered LDL within the intima, consistent with the notion that E-LDL represents a complement-activating entity in the early lesion.

**Methods**

**LDL and LDL Derivatives**

LDL (d = 1.025 to 1.052 g/mL) was isolated from fresh, nonlipemic human plasma by a modified method43 based on preparative density gradient ultracentrifugation. LDL was stored in the presence of 0.5 mmol/L EDTA for a maximum of 4 weeks. None of the regular blood donors had diabetes mellitus or treatment for arterial hypertension. Oxidative and enzymatic modifications of LDL were undertaken essentially as described.44 For oxidation, dialyzed LDL solutions (0.3 mg/mL cholesterol) were incubated with 50 μmol/L CuSO4 for 16 hours at 37°C. The content of thiobarbituric acid–reactive substances ranged between 50 and 60 mmol/mg per mg cholesterol. For enzymatic modification, LDL was treated with trypsin, cholesterol esterase, and neuraminidase according to the previous protocol.42 E-LDL preparations contained no detectable amounts of oxidized lipids, as revealed by determination of lipid hydroperoxides and thiobarbituric acid–reactive substances. LDL cholesterol content was determined with a commercial kit (Boehringer-Mannheim), and protein content was analyzed by using the Bradford assay (Roth).

E-LDL was isolated from atherosclerotic plaques as previously described.35 LCA and E-LDL had similar properties:42 in the electron microscope, they appeared to comprise a heterogeneous mix of fused LDL particles. They exhibited a pronounced negative charge in agarose gel electrophoresis and spontaneously activated complement via the alternative pathway.

**mAbs Against LDL and E-LDL**

Mouse mAbs were generated by immunization of BALB/c mice with 50 μg native human LDL or with E-LDL suspended in Quil A adjuvant. Booster injections of each of the antigens (20 μg) in Quil A were performed on days 20 and 40 after initial immunization. Thereafter, the respective antigens (20 μg) dissolved in isotonic saline were given intraperitoneally on days 54, 55, and 56. Four days later, spleen cells were harvested and fused with X63-Ag8.653 myeloma cells, as described.43 Hybridoma cultures were screened for antibodies by ELISA. Colonies producing antibodies reactive with E-LDL only were selected as being specific for a neoantigen. Two colonies producing antibodies against E-LDL and one colony with antibodies directed against LDL were cloned by three cycles of limiting dilution. Then the antibodies were purified from the culture supernatant by affinity chromatography on protein A-Sepharose. The mAbs specific for neoepitopes of E-LDL (AIL-2, AIL-3) and the antibody directed against native LDL (AIL-1) were of the IgG1 subclass. All antibody preparations were adjusted to a protein concentration of 1 mg/mL.

**Determination of Antibody Specificity**

Antibody specificity was determined in a standard assay using 96-well Maxisorb plates (Nunc), which were coated overnight at 4°C with LDL, E-LDL, ox-LDL, or LCA (isolated from early fatty streaks of human aorta) at 10 μg cholesterol/mL, pH 9.6. As a standard negative control, plates were also coated with the modifying enzyme mix (trypsin, cholesterol esterase, and neuraminidase). The plates were washed twice in TBS and blocked with TBS containing 0.3% Tween-20, 150 mmol/L NaCl, and 20 mmol/L Tris (pH 7.5) for 30 minutes at room temperature. Thereafter, the mAbs were serially diluted in TBS containing 0.05% Tween-20, 150 mmol/L NaCl, and 20 mmol/L Tris (pH 7.5) to protein concentrations of 0.04 to 20 μg/mL. Antibody dilutions were added for 1 hour at room temperature. After washing twice in TBS supplemented with 500 mmol/L NaCl and 0.05% Tween-20 (pH 7.5), the second rabbit anti-mouse biotinylated antibody (1:1000) was added and incubated for 60 minutes at room temperature. After another washing, streptavidin-biotinylated horseradish peroxidase complex (Amersham-Buchler) was added for 45 minutes at 37°C. The reaction was developed with tetramethyl benzidine in citrate buffer (40 mmol/L citrate, 10 mmol/L KCl, 135 mmol/L NaCl, pH 5.0). The reaction was stopped after ~15 minutes by addition of 1.8N H2SO4, and the absorbance was determined on an ELISA plate reader at 450 nm using a reference filter set to 620 nm.

Specificities of mAbs were confirmed as follows. LDL (1 mg cholesterol/mL) was coupled to cyanogen bromide–activated Sepharose beads (Pharmacia) following the standard protocol, and solid-phase LDL was subjected to triple–enzyme modification. Thereafter, the Sepharose beads were washed twice in PBS and incubated in the presence of 0.1 mg/mL soybean trypsin inhibitor (Sigma Chemicals) for 1 hour at room temperature. Then the Sepharose beads were washed twice in 10 volumes of TBS (Tris-HCl 20 mmol/L, NaCl 150 mmol/L, Tween-20, 0.05%, pH 7.5). For absorption studies, an mAb was added to 0.5 mL of a 20% LDL-Sepharose suspension at a final antibody concentration of 1 μg/mL for 2 hours at room temperature. The Sepharose was removed by centrifugation, and the supernatants were tested by ELISA and immunohistochemistry.
SDS Polyacrylamide Gel Electrophoresis and Western Blot Analysis

Samples (20 μg cholesterol per lane) were separated in 9% SDS polyacrylamide gels. The proteins were transferred to nitrocellulose membranes that had been treated with methanol followed by CAPS buffer (10 mmol/L 3-cyclohexylamino-1-propane sulfonic acid, 10% methanol, pH 10.5) for 1 hour at 1 mA/cm2. The nitrocellulose membranes were washed three times with PBS/0.05% Tween-20 and blocked with PBS containing 1% Tween-20 and 5% BSA for 1 hour at room temperature. After two washes in PBS/0.05% Tween-20, membranes were incubated with the respective mAbs diluted 1:2,000 overnight at 4°C. The biotinylated sheep anti-mouse antibody (Amersham) was diluted 1:1,000 in PBS/Tween-20 0.05% and added for 60 minutes at room temperature. Subsequently, streptavidin biotin–conjugated horseradish peroxidase (1:1,000 in PBS) was added for 1 hour at room temperature. After another two washes in PBS/0.05% Tween-20 and two washes in PBS, reactions were developed by using diaminobenzidine containing 0.002% mmol/L H2O2 and CoCl2. Once the bands were clearly visible, the membranes were rinsed in water and dried.

Coronary Artery Specimens

Specimens of coronary arteries were prepared from ~500 hearts obtained at autopsies. They were fixed in 4% buffered formalin, embedded in paraffin, sectioned, and stained with hematoxylin and eosin and van Gieson’s staining. Ten specimens of intimal atherosclerotic lesions and fatty streaks fulfilling the stringent criteria for early lesions were selected from 10 vessels for analysis. Serial transverse sections (4 to 5 μm) were cut and used for immunohistochemistry.

Antibodies

mAb AIL-1 was used at a 1:200 dilution; mAbs AIL-2 and AIL-3 were usually used at a 1:400 dilution. In later experiments, 1:5,000 dilutions were found to give similar staining. Terminal C5b-9 complement complexes were detected by a murine mAb (clone 978/394, IgG2a, used at a 1:200 dilution) recognizing a neoepitope of C5b-9. The murine mAbs PG-M1 (IgG2a) and KPI (IgG2a), both used at a 1:100 dilution and directed against the macrophage marker CD68, were purchased from Dako. Primary antibodies were detected by using biotinylated anti-mouse polyclonal antibodies (Vector Laboratories).

Immunohistochemistry

Serial sections were deparaffinized in xylene. After blocking of endogenous peroxidase activity with 3% H2O2, sections to be probed with antibodies against the macrophage marker CD68 were predigested in 0.1% pronase E solution for 20 minutes. Pronase digestion was omitted when anti-LDL antibodies were tested. After blocking with 5% normal horse serum, the slides were incubated with the primary antibody for 1 hour at room temperature, followed by incubation with biotin–conjugated horseradish peroxidase for 30 minutes and with avidin biotin peroxidase reagent for 45 minutes at room temperature. Reaction products were visualized by immersion in diaminobenzidine tetrachloride (brown color deposits). Finally, the slides were counterstained with hematoxylin and mounted.

Double-Staining Immunoperoxidase Method

Double staining for C5b-9 and E-LDL, or for E-LDL and macrophages, was performed as follows. Slides were incubated with the first antibody against the neoantigens of the terminal C5b-9 complement complex or E-LDL, respectively, visualized by immersion in diaminobenzidine tetrachloride (for details, see above), and then rinsed in TBS. Following renewed blocking with 5% normal horse serum, slides were incubated with one of the two primary antibodies against E-LDL or the macrophage marker CD68, respectively. Slides were then incubated with biotin-conjugated anti-mouse antibody followed by avidin biotin peroxidase reagent, and the reaction products were visualized by immersing the slides in 3-amino-9-ethylcarbazole (red color deposits). Finally, the slides were counterstained with hematoxylin and mounted.

Results

Specificities of mAbs AIL-2 and AIL-3

Fig 1 depicts the results of ELISAs conducted with mAbs AIL-1, AIL-2, and AIL-3 using LDL, ox-LDL, E-LDL, and LCA as antigens. Positive reactions were observed with mAb AIL-1 in all cases, whereas mAbs AIL-2 and AIL-3 recognized E-LDL and LCA, but not native LDL or ox-LDL. The ELISA reactivity of LCA preparations was lower than that observed with freshly prepared E-LDL, and the strongest signals with LCA were obtained with mAb AIL-3. mAbs AIL-1 and AIL-2 produced weak but still clearly positive signals (A450 0.15 to 0.25) over background (A450 ≤ 0.05). When E-LDL preparations were aged at 4°C for 8 to 12 weeks, ELISA signals generated with mAbs AIL-1 and AIL-2 declined (not shown). We interpreted this to indicate that progressive degradation leads to slow reduction in the epitopes recognized by mAbs AIL-1 and AIL-2. The ELISA patterns were reproduced with four different preparations of each antigen. No positive signals were observed when an isotype-matched, irrelevant mAb was used.

When LDL was subjected to single-, double-, or triple-enzyme treatments, it was found that the neoepitope reacting with mAb AIL-2 indeed became exposed already after trypsin attack, though not after a single treatment with either cholesterol esterase or neuraminidase (Fig 2). Combined treatment with trypsin plus cholesterol esterase led to enhanced reactivity in the ELISA, independent of additional treatment with neuraminidase. In contrast, the neoepitope reacting with mAb AIL-3 became exposed only after combined treatment of LDL with trypsin and cholesterol esterase. None of the mAbs reacted with the enzyme mix alone.

The reliability of the ELISAs was confirmed by antibody absorption experiments. When mAb AIL-2 was incubated with solid-phase, native LDL, no antibody binding to the Sepharose occurred, and the supernatant remained ELISA-
positive. When the antibody was incubated with enzymatically modified, solid-phase LDL, binding to the Sepharose occurred, and ELISAs conducted with the supernatant became negative (data not shown). The corresponding results of immunohistochemical staining are depicted below.

Results of SDS–polyacrylamide gel electrophoresis and Western blotting are shown in Fig 3. Intact apo-B was recognized by mAb AIL-1, but staining with mAb AIL-2 or AIL-3 was almost imperceptible. After enzyme treatment, mAb AIL-1 recognized many apoB fragments, indicating reactivity with a linear epitope that is retained in many degradation products, whereas mAb AIL-2 reacted mainly with one fragment of $M_r \approx 60,000$, and mAb AIL-3 reacted mainly with a doublet of $M_r 68,000$ and $75,000$.

To determine whether the mAbs would exhibit the corresponding specificities when employed in immunohistochemical studies, LDL, ox-LDL or E-LDL was injected into the wall of an infant aorta that had been freshly obtained at autopsy and that was devoid of atherosclerotic lesions. mAb AIL-1 stained native and ox-LDL (Fig 4A and 4E), whereas no staining of either antigen was observed with mAb AIL-2 or AIL-3 almost imperceptible. After enzyme treatment, mAb AIL-1 recognized many apoB fragments, indicating reactivity with a linear epitope that is retained in many degradation products, whereas mAb AIL-2 reacted mainly with one fragment of $M_r \approx 60,000$, and mAb AIL-3 reacted mainly with a doublet of $M_r 68,000$ and $75,000$.

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**E-LDL Is Present in Early Human Atherosclerotic Lesions**

Ten specimens of initial atherosclerotic lesions and fatty streaks fulfilling the criteria of early lesions as defined by Stary were examined, and similar findings were made in all cases. The general morphology of the lesions has been described. The early lesions were all within diffuse, adaptive, intimal thickening consisting of a fibromuscular layer at the base of the intima adjacent to the internal elastic lamella and a fibroelastic layer bordering the lumen (Fig 5A). The lesions themselves were characterized by macrophages either appearing as isolated groups of round or spindle-shaped cells within the intima or forming one or more layers next to the luminal surface (Fig 5B). Occasionally, they were visible throughout most of the intima. With the use of specific mAbs, E-LDL was detectable in every early lesion examined, whereby mAbs AIL-2 and AIL-3 generated similar staining patterns. With mAb AIL-1, weaker staining was observed in identical areas (Fig 6A and 6B), suggesting that the bulk of LDL in the lesions was modified. Typically, there was predominant, focal deposition of E-LDL in the insudative zone below the layer of macrophage foam cells (Figs 5C and 6) and in the deeper part of the intima adjacent to the media, sometimes also filling the layer to the level of the luminal surface and thus intermingling with macrophages. Nondiseased regions within diffuse, adaptive, intimal thickenings did not show staining with any antibody (Fig 5D).

E-LDL was localized mainly extracellularly. This is exemplified in Fig 7, which shows staining of parallel sections with van Gieson’s stain (to demonstrate connective tissue) and mAb AIL-2. The extracellular matrix was suffused with lipid that caused distension of the tissue fibers, and positive staining for E-LDL was always seen within these areas. On occasion, E-LDL appeared to be localized within macrophage foam cells. This was especially apparent in the more superficial areas of the subendothelium (not shown).
When mAb AIL-2 was preabsorbed with solid-phase native LDL, immunohistochemical staining remained positive. When the antibodies were preabsorbed with solid-phase E-LDL, the immunohistochemical staining became negative (not shown). E-LDL was also detectable in atherosclerotic lesions of coronary arteries obtained from explanted hearts (Fig 8A). Because these specimens were immediately fixed in 4% buffered formalin after removal, the detection of E-LDL dispelled concerns that staining might represent a postmortem artifact. As a final control, native LDL was injected into a nonatherosclerotic artery wall, and the specimen was left unfixed at room temperature for 3 days. Staining was observed with mAb AIL-1 (Fig 8B) but not with mAb AIL-2 or AIL-3 (Fig 8C).

**Colocalization of C5b-9 With E-LDL**

C5b-9 deposits were present in every atherosclerotic lesion. The pattern of C5b-9 deposits has been described in detail. The predominant manifestation of C5b-9 was a deposition of small granules in the insudative zone below the layer of macrophage foam cells and in the deeper part of the intima adjacent to the media. On occasion, a more diffuse deposition extending over the whole width of the intima was observed. The controls processed with the irrelevant isotype-matched monoclonal mouse antibody instead of the specific antibody were completely negative.

More precise information on the spatial relation between E-LDL and C5b-9 was obtained by double staining. There was a colocalization of C5b-9 with E-LDL within the intima, whereby as a rule, a more extensive area was occupied by E-LDL than by the terminal complex (Fig 9A). Most significantly, deposition of the terminal complex was never seen in the absence of E-LDL within the intima.

Double staining for macrophage antigens and E-LDL was also performed, and these experiments corroborated the above findings. In particular, it was evident that E-LDL was predominantly deposited in the insudative zone below the layer of macrophage foam cells (Fig 9B) and in the deeper part of the intima adjacent to the media.

**Discussion**

The point at which our hypothesis on the initiation of atherosclerosis departs from the conceptual mainstream of research relates to the nature of the predominant LDL modification that renders the molecule atherogenic. While it is not contested that oxidation may play a role, failure to biochemically detect ox-LDL in large quantities in plaque material suggests to us that this LDL derivative conceivably plays its harmful part predominantly at the early phase of contact with and insudation into the vessel lining. Then, it would be unnecessary to hypothesize that LDL must always be oxidized to become atherogenic. Instead, endothelial cells may be stimulated by only a minor oxidized lipoprotein fraction, with the bulk of LDL codiffusing into the subendothelium in native form. This would stand in accord with the recent data of Kühn et al, who found that only approximately 1 in 1000 to 2000 fatty acid molecules is oxidized in early human lesions. We envisage the key alterations to occur after insudation through...
degradation by enzymes that could be ubiquitously present at low concentration in tissues. Proteases and cholesterol esterase are major lysosomal constituents that are needed by cells to degrade endocytosed LDL. That small amounts of these enzymes may be continuously liberated appears to us likely, and we tacitly assume that they can initiate E-LDL formation. In vitro, neuraminidase is additionally required to transform LDL to an entity resembling plaque lipids. Desialylated LDL has actually been detected in the blood of patients with atherosclerosis, and treatment of LDL with neuraminidase promotes lipoprotein aggregation. Should this enzyme indeed play a role in vivo, its source would remain to be determined. Already at the stage of modification with protease and cholesterol esterase, LDL acquires the propensity to avidly interact with extracellular matrix components, which would hasten its fate to remain entrapped in the subendothelium.

Figure 4. Demonstration of neoepitope recognition by mAbs AIL-2 and AIL-3 by immunohistochemistry. Sequential sections were stained with mAbs AIL-1, AIL-2, and AIL-3; identical results were obtained with the latter two antibodies. Native LDL, E-LDL, or ox-LDL was injected into the vessel wall of the thoracic aorta of a 5-year-old child prior to processing of the tissue. The lumen is to the bottom. A, Native LDL stained with mAb AIL-1; B, native LDL stained with mAb AIL-2; C, E-LDL stained with mAb AIL-2; D, ox-LDL stained with mAb AIL-2; E, ox-LDL stained with mAb AIL-1; F, ox-LDL stained with mAb AIL-3. Scale bars indicate 50 μm.

Figure 5. Sequential sections of an early atherosclerotic lesion (A through C) and a nondiseased region (D) within an adaptive intimal thickening of a human coronary artery. The lumen is to the lower right-hand corner. A, α-Actin stain, demonstrating the preponderance of smooth muscle cells in the fibromuscular layer at the base of the intima (arrowheads); B, macrophage marker CD68 stain, demonstrating macrophage foam cells forming a layer next to the luminal surface (arrowheads); C, E-LDL stain with mAb AIL-3, demonstrating deposition of E-LDL predominantly in the insudative zone below the layer of macrophage foam cells; D, E-LDL stain of a nondiseased region (atherosclerosis-free intima, same specimen as shown in C) demonstrating the specificity of the antibody. The demarcation between intima and media is indicated by an arrow. Scale bars indicate 25 μm.
particles,35 similar to those that had also been described in other laboratories.30–34 These lipid particles contained large amounts temporally associated with lipid deposition.41 A directed search conducted with human material then led to the isolation of LCA, a lesion lipid endowed with complement-activating properties as LCA and found that E-LDL, through its recognition by a macrophage scavenger receptor, was a potent inducer of foam cell formation.42 Foam cell induction by extracellular lipoprotein causing distension of the tissue fibers and media is indicated by an arrow. Scale bars indicate 50 μm.

Before the present investigation was launched, our hypothesis had been based on two converging lines of evidence. On the one hand, we had shown that complement activation occurs at an early stage of lesion development in rabbits, espoused by Steinbrecher and Lougheed.28 On the other hand, we discovered that LDL modified enzymatically in vitro (i.e., E-LDL) displayed the same salient properties as LCA and found that E-LDL, through its recognition by a macrophage scavenger receptor, was a potent inducer of foam cell formation.42 Foam cell induction by lesion-derived lipids distinct from ox-LDL had been independently demonstrated by Steinbrecher and Lougheed.28 On these grounds, E-LDL logically emerged as a novel candidate to assume a relevant role in atherogenesis.

Acquisition of direct evidence that E-LDL is indeed present extracellularly in early atherosclerotic lesions became a pressing issue, and immunohistochemical localization appeared to us most straightforward. mAbs against ox-LDL have been described and employed in several immunohistological studies. In contrast, mAbs recognizing E-LDL, but not ox-LDL, have not been available hitherto. Two antibody clones were here identified, directed against apoB epitopes that are concealed in native LDL and ox-LDL but that become exposed when the lipoprotein is enzymatically degraded. By direct injection of LDL into arterial walls, we ascertained that these antibodies would not stain native LDL or ox-LDL, and thus, excluded the possibility that the lipoprotein might be rendered reactive by the immunohistochemical staining protocol. Because the antibodies could be used on paraffin-embedded sections, a collection of preserved tissues could be screened, and a large number of well-defined early lesions was studied. Extensive deposits of E-LDL were detected without exception, and never was its presence observed in nonatherosclerotic areas. Detection of deposits in atherosclerotic lesions of coronary arteries from two explanted hearts dispelled concerns that staining might represent a postmortem artifact. In many early lesions that contained very few infiltrating cells, it was evident that E-LDL was located extracellularly. If we may assume that fusion and enzymatic degradation are essentially synonymous, our findings are in perfect accord with the freeze-etch electron micrographs of Frank and Fogelman,34 showing extracellular fusion of LDL particles in rabbit early lesions.

The neoepitopes recognized by the two mAbs appear to become exposed at different stages of enzymatic lipoprotein degradation: mAb AIL-2 recognizes LDL after proteolytic nicking alone, whereas mAb AIL-3 reacts only after combined treatment with a protease and cholesterol esterase. It is known that LDL treatment with cholesterol esterase renders cryptic sites in apoB accessible for proteases.54 The reactivity of mAbs AIL-3 against both E-LDL and LCA is admittedly weak in the ELISA. However, mAb AIL-3 stains lesion lipoproteins as strongly as mAb AIL-2 in histological sections, so it is our bias that the ELISA results are essentially trustworthy. Both antibodies generated similar staining patterns, lending credence to the contention that LDL contained in early lesions becomes modified extracellularly by at least two enzymes at very early stages in lesion development. It is noted that both mAbs AIL-2 and AIL-3 recognize proteinaceous neoepitopes of apoB and that ELISA reactivities became weaker upon prolonged aging of E-LDL. This could indicate slow, progressive destruction of the epitope and would also explain the poor (but still significant) reactivity of LCA. Neoepitope destruction would be expected to proceed rapidly after uptake of E-LDL by macrophages; this may be the reason why positive staining for E-LDL was only sporadically seen in the cells. Experiments are currently underway to examine this possibility.

The data showing colocalization of C5b-9 deposits with E-LDL are compatible with the assumption that lesion LDL is a major complement-activating moiety. Single- or even dou-
ble-enzyme treatment does not suffice to transform LDL in vitro to a complement-activating moiety. Thus, it seems reasonable that C5b-9 deposits reflect the presence of extensively modified LDL, whereby other possible complement-activating mechanisms are not excluded. While these considerations relate to a possible role for C5b-9 as a marker of extensive enzymatic LDL modification, the possible significance of local complement activation itself can hardly be overemphasized. Given the multifaceted proinflammatory processes that are driven along the entire complement pathway, the possibility that these may contribute toward lesion development appears imminent.

Let us now compare our immunohistochemical findings with the literature on ox-LDL. Immunohistochemical analyses of atherosclerotic lesions have been performed on rabbit and human tissues. Additionally, there is one study on focal glomerulosclerotic lesions in the rat. In considering the available data, it is important to heed two questions. First, was the work conducted on early lesions prior to massive cellular infiltration, and were the LDL deposits extracellular or intracellular? Macrophages produce reactive oxygen metabolites, so detection of ox-LDL in cells may not attain the significance attributed to extracellular, modified LDL. Second, was human material examined?

In Witztum’s group, polyclonal antibodies and mAbs against apolipoprotein B-fragments were used to detect ox-LDL deposits in older lesions, and these deposits were mainly cell-associated. Hammer et al similarly showed cellular staining for ox-LDL in macrophage foam cells in one cryosection of human atherosclerotic tissue from the femoral artery in a case of advanced arterial occlusive disease. In rabbit studies by Haberland et al and Boyd et al, monoclonal antibodies against MDA-LDL were used to demonstrate extracellular deposition of MDA-LDL adducts in early lesions. To our knowledge however, those monoclonal antibodies were never employed to show similar extracellular distribution of MDA-LDL in early human lesions. Studies from the laboratory of Steinberg and Witztum, conducted with either poly- or monoclonal anti-MDA-LDL antibodies, showed primarily cellular association of ox-LDL in more advanced human and rabbit lesions. Finally, Steinbrecher’s group also reported cellular localization of ox-LDL in experimental focal glomeronephritis of the rat.

Thus, none of these studies has demonstrated extensive extracellular deposition of oxidized LDL in the early human atherosclerotic lesion. This contrasts with data of Jürgens et al and Napoli et al who provided positive immunohistochemical evidence with the use of other monoclonal antibodies. The causes for the apparent discrepancies between the different studies are unclear, and it will be of interest to determine whether certain antibodies raised against ox-LDL cross-react with E-LDL neoepitopes. In any event, the present study
provides evidence for the presence of extracellularly degraded LDL in early lesions, a finding that represents a fundamental piece of evidence to support our hypothesis on the pathogenesis of atherosclerosis in the human organism.

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References


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