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In Situ Immunolocalization of Lipoproteins in Human Arteriosclerotic Tissue

Birgit Kaesberg, Bärbel Harrach, Hans Dieplinger, and Horst Robenek

The concentrations of serum lipoproteins, especially those of low density (LDL) and high density (HDL) lipoprotein, are related to the pathogenesis of arteriosclerosis. However, there is a lack of data concerning lipoprotein distribution in the human arteriosclerotic plaque. To detect these lipoproteins, we performed immunogold labeling on ultrathin sections of fixed and embedded human arteriosclerotic tissue. We used a panel of specific antibodies to different lipoproteins and their apolipoprotein constituents, namely LDL, formaldehyde-fixed LDL, apolipoprotein B-100, HDL, and formaldehyde-fixed apolipoprotein A-I. We also applied antibodies to α-actin and cathepsin D to characterize the cells and organelles involved in lipoprotein uptake and metabolism. Semiquantitative evaluation was carried out for a detailed comparison of the results obtained. Electron microscopic examination revealed that the majority of HDL and LDL in the pathological tissue was localized intracellularly in macrophage-derived foam cells and smooth muscle cells, whereas only LDL was found in the extracellular matrix. In some cases, we observed an intracellular accumulation of lipoproteins in electron-dense vesicles, which appeared to be of lysosomal origin, as shown by double labeling with an antibody to cathepsin D. These vesicles were present only in macrophage-derived foam cells, which were localized in the necrotic cores of arteriosclerotic plaques, and could not be found in healthy tissue or in the early stages of arteriosclerotic disease. (Arteriosclerosis and Thrombosis 1993;13:133–146)

Key Words • arteriosclerosis • immunocytochemistry • foam cells • immunolocalization of lipoproteins

Hypercholesterolemia is the most clearly recognized risk factor in the pathogenesis of arteriosclerosis.1–6 The arteriosclerotic lesion in humans is characterized by the accumulation of lipids in and around the cells of the intima. A number of mechanisms have been postulated, on theoretical grounds and from experimental studies, to account for the role of lipids in lesion formation. Lipid-filled foam cells are a major cellular component of arteriosclerotic plaques.7,8 Foam cells are now known to originate largely from monocytes that enter the intima and become transformed into macrophages, although smooth muscle cells (SMCs) also seem to have an ability for lipid accumulation.9 Low density lipoproteins (LDLs), β–very low density lipoproteins, and modified LDL10–16 promote foam cell formation. High density lipoproteins (HDLs) have been postulated to be involved in the process of reverse cholesterol transport.17–20 According to this hypothesis, HDL picks up cholesterol from various tissues and transports this cholesterol, either directly or indirectly (via transfer to other lipoproteins), to the liver for excretion from the body.

Nearly all data concerning the function and role of lipoproteins in the pathogenesis of arteriosclerosis have been generated from in vitro experiments with different cell culture systems. However, data on the presence of lipoproteins in the arteriosclerotic plaque are controversial, and only a little is known about the location of lipoproteins in and around the cells of the lesion in situ.21–25 Previous morphological studies performed at the light microscopic level have left unresolved the question as to how the various lipoproteins are distributed and organized in relation to one another in the different compartments of the plaque. Knowledge about the exact location of these molecules within the lesion may give further insights into the composition of the plaque and the function of lipoproteins in the development and/or regression of the plaque.

In the present study, we have combined high-resolution immunoelectron microscopy with a panel of polyclonal antibodies that are specific for the location of lipoproteins and apolipoproteins at the ultrastructural level. Using antibodies against native and formaldehyde-fixed LDL and apolipoprotein (apo) A-I, we compared the influence of the fixation routine on the antigenic recognition of lipoproteins in an enzyme-linked immunosorbent assay (ELISA) and the embedded tissue.

Methods

Chemicals

Lowicryl K4M was purchased from Plano, Marburg, FRG. Protein G–gold complexes were purchased from...
Antibodies

In this study, we used polyclonal antisera to human β-lipoproteins (anti-LDL) and human α-lipoproteins (anti-HDL) raised in the goat (Sigma, Munich, FRG) and goat anti-human apo B-100 (Tago, Burlingame, Calif.). The polyclonal antibodies to formaldehyde-fixed human apo A-I (anti-fix apo A-I) and fixed human LDL (anti-fix LDL-BK) were prepared in our laboratory by following the protocol described by Harrach and Robenek.26 Another polyclonal antibody to formaldehyde-fixed LDL (anti-fix LDL-D) raised in the rabbit was kindly provided by Dr. H. Dieplinger, University of Innsbruck, Innsbruck, Austria. For the characterization of SMCs in the arteriosclerotic plaques, we used the mouse monoclonal antibody asm-1 raised to α-actin of SMCs (anti-SMC), which was purchased from Progen, Heidelberg, FRG. Polyclonal rabbit anti-human cathepsin D affinity-purified antibodies were prepared and characterized by von Figura, Hasilik, and coworkers (Schulze-Lohoff et al27 and Gupta et al28) and were kindly provided by Dr. A. Hasilik, Institute for Physiological Chemistry, University of Münster, Münster, FRG. For the secondary antibodies, we used rabbit anti-goat or rabbit anti-mouse immunoglobulin Gs purchased from Dianova, Hamburg, FRG.

ELISAs

ELISAs were performed to test the specificity of the antibodies used. LDL (d=1.019--1.063 g/ml) and lipoprotein-deficient serum (LPDS) (d>1.250 g/ml) were isolated from fresh human serum of individual normolipidemic volunteers by sequential ultracentrifugation.29 The protein content of the different lipoprotein fractions was determined by the method of Lowry et al30 with bovine serum albumin (BSA) as the standard.

Lipoproteins (10 ng protein per well) were diluted in 100 μl of 50 mM Na2CO3 buffer, pH 9.6, with 0.2% NaN3 and were coated as the substrate on microtiter plates (Nunc, Roskilde, Denmark) for 12 hours at 37°C. Plates were then washed three times with phosphate-buffered saline (PBS) and saturated at 37°C for 2 hours with 100 μl of 1% BSA in PBS. After washing with PBS containing 0.05% Tween 20 (PBST), the plates were then incubated with 100 μl of the antibody (serially diluted in PBS) with 1% BSA for 1 hour at 37°C. Unbound antibodies were removed by washing with PBST. The plates were then incubated with peroxidase-conjugated secondary antibodies (1 hour at 37°C). After washing with PBST, we added 100 μl of freshly prepared substrate solution (40 mg orthophenylenediamine in 100 ml of 0.1 M phosphate-citrate buffer, pH 5.0, containing 0.025% H2O2) to each well and then incubated the plates in the dark for 30 minutes at 37°C. The reaction was stopped by adding 2.5 M H2SO4 (50 μl per well), and the absorbance of each well was determined at 492 nm.

For the competitive ELISA, a fixed dilution of primary antibody was used, which contained increasing amounts of prefixed LDL as the competitor in the dilution buffer consisting of PBS with 1% BSA.

Tissue Procurement

Severe arteriosclerotic plaque tissues from human femoral arteries obtained at surgery were kindly provided by Dr. E. Vollmer, Institute for Pathology, University of Münster, Münster, FRG. In the present study, two samples of straight arterial segments containing arteriosclerotic plaques were included from three patients, two female donors (60 and 63 years old) and one 65-year-old man donor. For this study, we have not yet correlated our results to risk factors that these patients may have exhibited, like plasma LDL and/or plasma HDL levels, smoking status, or blood pressure. As a control, we used arterial tissue obtained at surgery from a 75-year-old man suffering from aortic valve incompetence due to pericarditis but without any sign of arteriosclerotic alterations.

Electron Microscopy

Tissue preparation for electron microscopy. Pale white spots on the luminal sides of the freshly harvested arteriosclerotic arteries were cut out with a scalpel and fixed in 4% formaldehyde diluted in PBS, pH 7.4, for 10 hours at 4°C. The tissue samples with an approximate size of 1 mm3 were dehydrated in an ascending series of dimethylformamide and embedded in Lowicryl K4M as described previously.31 The tissue blocks were orientated in such a way as to facilitate sectioning of the intima. Ultrathin sections of

**FIGURE 1.** Overview photomicrograph at the light microscopic level shows the nature of the lesions studied. In the intima of arteriosclerotic human arteries, macrophages (M0) transformed to lipid-rich foam cells, smooth muscle cells (thin, dark feature just above “SMC” designation), and cholesterol crystal deposits (CC) were found. Bar=30 μm.
FIGURE 2. Electron photomicrograph of human artherosclerotic plaque tissue. Application of the antibody to α-actin resulted in specific labeling of the cytoplasmic ground substance of the smooth muscle foam cells (SMC). Macrophage foam cells (MØ) and the abundant extracellular matrix (ECM) between cells are devoid of gold particles, which emphasize the specificity of this antibody. Bar=1 μm.

the embedded tissues were cut on a Reichert ultramicrotome OmU2 with a diamond knife and picked up on Formvar-coated copper grids (150 mesh) for immunogold labeling as described below.

Immunogold Electron Microscopy

Preparation of colloidal gold and protein A–gold complexes. Monodisperse gold sols of 12-nm diameter were prepared by reduction of chloroauric acid with sodium citrate as the reducing agent.32 Gold particles (8 nm) were prepared with tannic acid/citrate.33 Protein A was linked to the gold particles by following the protocol of Roth et al.34 The gold sols could be stored at 4°C in PBS/1% BSA/0.02% NaN₃ for several weeks with no loss of immunoreactivity. Before use, the conjugates were briefly centrifuged to remove aggregated gold particles (2,500g for 12-nm particles, and 4,000g for 8-nm particles) and were routinely examined by negative staining.

Postembedding immunogold electron microscopy. Immunocytochemical labeling of the thin sections was performed at room temperature by floating the grids serially, section side down, on 20-μl droplets placed on wax sheets as follows: 1) Pretreatment to quench any aldehydes present on the section surface was performed with 1% BSA in PBS (pH 7.4) for 15 minutes followed by two 5-minute washes in PBS. Additionally, we incubated the sections for 15 minutes in 1% milk powder diluted in PBS, pH 7.4, to eliminate nonspecific binding. 2) After this pretreatment, the grids were placed on the drops with an appropriate dilution of the primary antibody containing 1% BSA, followed by three 5-minute washes in PBS. The dilution of the primary antibodies was tested on the tissue. Optimal dilutions were selected on the basis of maximal specificity and minimal background staining. The dilutions of the primary antibodies were made as follows: anti-LDL, 1:750; anti-apo B, 1:750; anti-fix LDL-BK, 1:20; anti-fix LDL-D, 1:100; anti-HDL, 1:100; anti-fix apo A-I, 1:30; anti-SMC, 1:75; and anti-cathepsin D, 1:300. 3) After removing the unbound primary antibodies by proper washing, the grids were floated on drops containing gold-labeled protein A or secondary antibodies, followed by three 5-minute rinses in distilled water. 4) Labeled grids were dried and stained with saturated aqueous uranyl acetate before examination in a Philips 410 electron microscope at 60 kV. All incubation steps with antibodies and protein A–gold were performed for 1 hour at room temperature. The specificity of immunostaining was checked by treating the grids as described above, but with nonimmune serum or the primary antibody omitted.
FIGURE 3. Electron photomicrographs showing label patterns obtained with commercially available antibodies to low density lipoprotein (panel A) and apolipoprotein B-100 (panels B and C). In both cases gold particles are found over the cytoplasmic ground substance of macrophages (MØ) and smooth muscle cells (SMC) as well as over the extracellular matrix (ECM) (panels A and B). Electron-dense organelles in the macrophages also reveal an intense labeling (panel C). Bars=1 μm.
To ensure that antigens were still recognizable after the entire embedding procedure, we performed single labeling as well as double-labeling experiments with anti-cathepsin D antibody as an internal standard. Double immunogold labeling was carried out by using a sandwich method on one side of the tissue sections. In this case we took advantage of the different origin of the antibodies and the heterogeneous reactivity of protein A and protein G with the immunoglobulin classes of various species. Labeling of the anti-LDL antibody raised in the goat was detected with protein G-gold conjugates with an average diameter of 12 nm, whereas the anti-
cathepsin D, generated in the rabbit, was detected with protein A conjugated to gold sols with an average diameter of 8 nm. The incubation steps were the same as for the single immunogold labeling described above. For a better estimation of cross reactions, we always performed the labeling of two antigens on one tissue section in the double-labeling experiments. These involved labeling with anti-LDL and protein G-gold first and then detection with anti-cathepsin D protein A-gold. The labeling was then carried out in the reverse order.

Semiquantitative evaluation. Twenty representative electron photomicrographs of the labeled tissue sections were used for the semiquantitative analyses, which were performed as follows. On each photomicrograph, different sample fields were placed over the selected subcellular compartments (e.g., the extracellular matrix, cytoplasm of SMCs and macrophages, and lysosomes of macrophages). The term “cytoplasm” is used to designate the cytoplasmic matrix of the cell, i.e., the “ground substance” between membranous organelles such as lysosomes. For most sampling, fields of 1 μm² were used; smaller fields were also used when necessary. The number of gold particles was counted in each field and was expressed as particle number per square micron. The mean value for each structure was determined and was expressed as a percentage of the total number of gold particles counted in each labeling experiment. We also performed Student’s t test to make certain that the
FIGURE 6. Electron photomicrographs showing labeling obtained with the antibody to cathepsin D. Panel A: Electron-dense lamellar organelles, which were found only in macrophages (M0) near the necrotic core of the plaque, reveal an intense and specific labeling. Panel B: Margins of lipid droplets (Li) in smooth muscle cells (SMC) also reveal labeling with anti-cathepsin D. Bars=0.5 μm.

Results

Immunocytochemical Characterization of Cellular Constituents of Arteriosclerotic Plaques

The specimens used in this study were characterized as advanced arteriosclerotic tissues with a notably thickened intima containing cholesterol crystal deposits. The lesions were lipid rich, and a necrotic core was present. Their cellular compositions consisted of macrophages, which were nearly all transformed into foam cells, and SMCs, as shown in Figure 1. Between the cells, abundant extracellular matrix was present (Figure 2). In the arteriosclerotic plaques we observed spindle-shaped SMCs (Figures 3, 4A, and 5B) with highly attenuated processes as well as SMCs containing large cytoplasmic lipid droplets and less extended processes (Figure 2). In both cell features, actin bundles could be observed. To confirm that these cells were SMCs, immunogold cytochemistry was performed to localize the SMC marker protein α-actin. This was done by postembedding immunoelectron microscopy with monoclonal antibodies to SMC α-actin. Figure 2 demonstrates the fact that only the SMC and not the macrophage is intensely labeled in a very specific manner with this technique.

The macrophage-derived foam cells, which were often arranged in the form of clusters in the arteriosclerotic tissue, were variable in shape, ranging from round to elongated. They could be distinguished from the SMCs by the abundance of relatively small cytoplasmic lipid droplets (Figures 2 and 3). Between the lipid droplets, electron-dense lamellar organelles were frequently present. Postembedding immunoelectron microscopy with monospecific antibodies to cathepsin D, a lysosomal marker enzyme (used as an internal standard, see "Methods"), resulted in an intense and specific labeling of these dense lamellar organelles (Figure 6A). In some macrophage-derived foam cells as well as in SMCs, we also detected labeling of cathepsin D at the margins of lipid droplets (Figure 6B).

Light microscopic observation of the control tissue confirmed that it had no intimal thickening or cholesterol deposits. Electron microscopic immunogold labeling with anti-SMC revealed that all cells in the specimen could be identified as SMCs. Neither macrophages nor signs of necrosis could be found in the control tissue. Some SMCs exhibited cytoplasmic lipid droplets, which were probably a manifestation of the advanced age of this patient.

Immunocytochemical Labeling of Lipoproteins

LDL. After immunogold labeling of the arteriosclerotic tissues with anti-LDL, gold particles were seen over the cytoplasmic ground substance of macrophages and SMCs as well as in the surrounding extracellular matrix (Figure 3A). Immunogold labeling of the arteriosclerotic tissue with the antibody to apo B-100 (Figure 3B) led to a pattern of labeling that was nearly identical to that obtained with the anti-LDL. Gold particles were also found over the electron-dense lamellar organelles in macrophages (Figure 3C). They were rarely seen over other organelles, such as mitochondria, or over the cisternae of the endoplasmic reticulum. In some cases, we observed a weak background labeling over either the nucleus or lipid droplets. We also found sparse labeling over endothelial cells and blood platelets. These results were emphasized by the semiquanti-
tative evaluation of the relative label intensities with these two antibodies, as shown in Figures 7b and 7d.

For the localization of LDL, we included two polyclonal rabbit anti-human LDL antibodies, which were produced to formaldehyde-fixed LDL (anti-fix LDL-BK and anti-fix LDL-D). After applying the anti-fix LDL-BK antibody, the label was mainly found over the extracellular matrix. The cytoplasm of the cells was also labeled, although less intensely than with the antibodies to LDL and apo B-100. Gold particles were particularly abundant over cytoplasmic electron-dense organelles in both SMCs (Figure 4a) and macrophages. With the anti-fix LDL-D antibody, not only did the extracellular matrix reveal an intense labeling but so too did the cytoplasmic ground substance of the macrophages (Figure 4b) and SMCs. Only a few electron-dense organelles in macrophages were labeled at their margins. Background labeling over the nucleus was seldom found and occurred to a lesser extent than in the antibodies to native LDL. The distribution of LDL established by the semiquantitative analyses in the different compartments together with that of HDL is shown in Figure 7.

**Figure 7.** Relative distribution patterns of gold particles over various cellular structures in the plaque tissue: extracellular matrix (ECM), smooth muscle cell (SMC), and macrophage (MØ) cytoplasmic ground substance, excluding organelles as well as lysosomes in macrophages (MØ-Lys). Panel a: Numbers of gold particles were counted in different sample fields and expressed as mean values of the number of gold particles per square micron. Student's t test revealed significant differences (p<0.01) in the number of counted gold particles over selected cellular compartments. Probability values larger than 0.05, determined to be not significantly different, are indicated in the figure (n.s.). Panels b–g: For these pie charts, mean values of the number of gold particles per square micron are expressed as a percentage of the total number of gold particles in each labeling experiment. For discussion, see text. Anti-LDL, antibody to low density lipoprotein; anti-apo B, antibody to apolipoprotein B; anti-fix. LDL-D and LDL-BK, antibodies to formaldehyde-fixed low density lipoprotein; anti-HDL, antibody to high density lipoprotein; anti-fix. apo A-I, antibody to formaldehyde-fixed apolipoprotein A-I.
FIGURE 8. Electron photomicrographs of control tissue showing that only a few gold particles diffusely distributed over the cytoplasm of the smooth muscle cell (SMC) are found after labeling with the antibody to formaldehyde-fixed low density lipoprotein. Extracellular matrix (ECM) is almost free of gold particles. Bar=1 μm.

The immunogold labeling of the control tissue with the anti-fix LDL-D antibody resulted in a weak and diffuse labeling of the SMC cytoplasm. The extracellular matrix was almost free of gold particles (Figure 8).

As a control for the labeling procedure itself, we omitted the specific primary antibody in the procedure. In addition, we performed the labeling procedure with nonimmune rabbit sera. In these cases gold particles could only be found very rarely, as shown in Figures 9A and 9B.

HDL and Apo A-I. After applying the polyclonal antibody to human HDL or formaldehyde-fixed apo A-I, the macrophages and SMCs revealed a labeling that was located over the cytoplasmic ground substance (Figures 5A and 5B). Labeling appeared to be most concentrated over the electron-dense lamellar bodies of macrophages (Figure 5A). Other components and organelles, such as the cisternae of the endoplasmic reticulum, lipid droplets, and mitochondria, were devoid of gold particles. The extracellular matrix appeared to be weakly labeled. Semiquantitative analyses confirmed that the relative distribution pattern of the gold particles over the various subcellular compartments in the plaque tissue for these two antibodies was nearly the same (Figures 7f and 7g).

The cytoplasmic ground substance of SMCs in the control tissue was as intensely and specifically labeled with the anti-fix apo A-I antibody as that of the cells in the pathological tissue (Figure 10).

Colocalization of LDL and cathepsin D. The double labeling revealed that both the internal-standard antibody to cathepsin D and the antibody to LDL were able to detect specific antigens in the embedded tissue.

However, the double labeling also showed that electron-dense organelles in macrophages contained both cathepsin D and LDL (Figure 11). Within these electron-dense organelles, LDL and cathepsin D did not display an identical distribution. The more electron-dense regions appeared to be exclusively labeled by the antibody to LDL (large arrows in Figure 11), whereas a relatively small part of the cathepsin D label was distributed over the dense cores. The majority of cathepsin D (small arrows in Figure 11) and LDL labels was randomly distributed over the rest of the electron-dense organelles. Lipid inclusions were completely devoid of label.

ELISAs

The results of the ELISA and competition tests with prefixed LDL as the competitor are shown in Figure 12. The antibody to native LDL specifically recognized LDL and to a lesser extent also recognized fixed LDL (Figure 12c). Human serum, which includes LDL, revealed a low detection signal. Both antibodies produced against prefixed LDL showed an improved recognition of fixed LDL compared with the antibody to native LDL. These antibodies exhibited a higher affinity to
FIGURE 9. Electron photomicrographs showing that the specificity of the immunostaining procedure itself was checked by omitting the primary antibody (panel A) and by performing the labeling with nonimmune sera (panel B). Gold particles were found only occasionally. ECM, extracellular matrix; MØ, macrophage. Bars=1 μm.
human serum than to LPDS, indicating their specificity (Figures 12a and 12b).

In addition, the specificity of the different antibodies to LDL was tested by competitive ELISA. The recognition of the coated prefixed LDL could be abolished by increasing the amounts of prefixed LDL as the competitor in the antibody dilution (Figure 12d). This could be shown more clearly with the antibodies to prefixed LDL than with the antibody to native LDL.

Discussion
The genesis and progression of arteriosclerosis are closely linked to an increased transport of plasma lipoproteins from the blood to the arterial wall. Conflicting data concerning the presence of lipoproteins in arteriosclerotic lesions have been reported in the literature. Morphological studies performed at the light microscopic level have been unable to ascertain the precise location of lipoproteins because of resolution limitations. Because there is a lack of data, we assumed that previous attempts to localize lipoproteins or apolipoproteins by immunoelectron microscopy so far have either failed or given unsatisfactory results.

One of the major problems of immunoelectron microscopic localization of proteins in general is the reduction of antigenicity caused by the fixation and embedding routines. In particular, the fixation routine often adversely influences antigenicity, resulting in a markedly reduced labeling intensity. Since it is generally impracticable to avoid these fixation-induced changes of antigenicity, we have tried to improve the lipoprotein/apolipoprotein recognition in fixed tissues by chemically modifying the lipoproteins/apolipoproteins with formaldehyde before immunization. Harrach and Robenek have demonstrated that antibodies against formaldehyde-fixed lipoproteins/apolipoproteins are more appropriate than are antibodies against unmodified lipoproteins/apolipoproteins for localization in fixed samples. The present study revealed that the antibodies generated against prefixed LDL are more appropriate because they exhibit a higher specificity and a lower background labeling on the tissue sections as well as on ELISA. In addition, the results of the competitive ELISA suggest a more specific binding between the fixed LDL and the antibodies to fixed LDL compared with the antibodies to native LDL.

Our data reveal that at the electron microscopic level, HDL, LDL, and the apop A-I and B-100 and/or their degradation products are present in several subcellular compartments of both macrophages and SMCs, as well as in the extracellular matrix of the arteriosclerotic lesions. The main differences between the results obtained with the various antibodies relate to the relative labeling intensities in the different compartments and are presented in Figure 7. Here it can be seen that all of the antibodies to unmodified lipoproteins used in this study (Figures 7b, 7d, and 7f) generated almost identical label intensities over the various cellular structures. Label intensities in the various compartments did, however, differ between the antibodies to formaldehyde-prefixed LDL (Figures 7c and 7e) compared with the antibody to formaldehyde-prefixed apo A-I (Figure 7g). In the case of the anti-fix LDL antibodies, the extracellular matrix was labeled to a much greater extent compared with the label with the anti-fix apo A-I or HDL antibodies.
In addition to the similar intense labeling of the extracellular matrix with the antibodies to fixed LDL, there was a discrepancy concerning the intracellular labeling with both of these antibodies (Figures 7c and 7e). This varying pattern of labeling suggests that the different antibodies to formaldehyde-fixed LDL that were used in this study recognized distinct antigenic determinants or perhaps different degradation products of the LDL molecules. Comparison of the relative label intensities of the antibodies to unmodified LDL and prefixed LDL showed that fixation with formaldehyde had a clear influence on the label pattern and thus also on the recognition of LDL in the fixed tissue. In the case of anti-HDL (Figure 7f) and anti-fix apo A-I (Figure 7g), these discrepancies were not found. We interpret this to mean that the effect of the fixative on the antigenic recognition of HDL is not as strong as for LDL.

Another possible reason for the discrepancy in the label pattern of the antibodies to LDL could be the oxidative state of the LDL used as an antigen for the generation of antibodies. Increased uptake of oxidized LDL by macrophages suggests that the antigenicity of the various epitopes of the LDL molecule could be affected. Furthermore, Lougheed et al demonstrated in vitro with macrophages that oxidized LDL is resistant to degradation by cathepsins. If this is also the case in the arteriosclerotic lesion, it would explain the accumulation of the LDL labeling in the electron-dense, cathepsin D-rich organelles. There are some hints in the recent literature that at least modest signs of oxidation could be shown with LDL isolated from human arteriosclerotic plaque tissue. For the generation of the antibody against fixed LDL, we used the LDL immediately after isolation. However, we did not test the recognition of oxidized LDL by the antibodies to the LDL used in this study.

In principle, our findings concerning the extracellular distribution of LDL are in agreement with previous investigations at the light microscopic level. Harrach et al reported that apo B-100 is located between the cellular components of the arteriosclerotic plaque. Niendorf et al found apo (a) and also apo B-100 in the extracellular matrix within the arterial wall. Mora et al classified the stages of plaque formation in hyperlipidemic arteriogenesis in the rabbit and observed that in the early stages, apo B-100 was associated with extracellular liposomes. However, the same authors pointed out that in the later stages of the lesion, apo B-100 was only occasionally visible intracellularly. In the necrotic cores of arteriosclerotic lesions we also found some electron-dense bodies within the extracellular matrix that were not associated with cells, which were labeled with the antibody to LDL. Perhaps these labeled compartments resemble the extracellular liposomes described by Mora et al.

The intracellular distribution of LDL observed contrasts with observations from light microscopy. We interpret our findings to be the result of the lysosomal degradation of LDL and the transport of cholesterol and amino acids to the cytoplasm. Because we used polyclonal antibodies for the location of the lipoproteins, it is likely that the antibodies also reacted with the degradation fragments of LDL; the antibodies would be expected to recognize these short-chain epitopes. There is one publication by Rosenfield et al that describes the intracellular labeling of oxidized LDL in SMCs in the necrotic cores of arteriosclerotic lesions in Watanabe.
FIGURE 12. Semilog plots showing specificity of antibodies that were checked by enzyme-linked immunosorbent assay (ELISA), as measured by the absorbance at 492 nm ($A_{492}$) of the peroxidase reaction product. Panels a–c: As the substrate, 10 ng formaldehyde-fixed low density lipoprotein (LDL) (●), native LDL (●), complete human serum (○), or lipoprotein-deficient serum (*) was used. Antibodies produced against prefixed LDL, anti-fix. LDL-BK (panel a), and anti-fix. LDL-D (panel b) reveal improved recognition of the fixed LDL compared with the antibody to native LDL (panel c). Panel d: Competitive ELISAs were performed at an antibody dilution of 1:8,000 for antibody to formaldehyde-fixed low density lipoprotein, anti-fix. LDL-D (●) and anti-fix. LDL-BK (○) and of 1:3,000 for anti-LDL (○). Recognition of coated prefixed LDL can be abolished by increasing the amounts of fixed LDL as the competitor in the dilution buffer of the primary antibody.

heritable hyperlipidemic rabbits, in which antibodies against oxidized LDL were used. Therefore, the antibodies to LDL used in the present study may also recognize epitopes of oxidized LDL.

In addition, we were able to locate LDL and HDL in electron-dense organelles of macrophages and in one case also of SMCs. By applying the antibody to cathepsin D, a marker enzyme of lysosomes that is routinely used as an internal standard, we have shown an intense labeling over these electron-dense organelles and have thereby identified them as lysosomes. Our double-labeling immuno-electron microscopy studies with antibodies to both cathepsin D and LDL have revealed that these organelles contain LDL and cathepsin D. The more electron-dense regions of lysosomes appeared to be exclusively labeled by antibodies to LDL. Lysosomes are subcellular compartments involved in the endocytotic process, and the intense labeling of LDL and HDL found over lysosomes reflects internalization and degradation activity. Our results suggest that LDL and HDL might be incorporated into the macrophages of arteriosclerotic plaques and hydrolyzed in a cathepsin D–rich lysosomal compartment. The localization of LDL and HDL in the same subcellular compartments suggests a common fate of both types of lipoproteins. Our results are consistent with biochemical studies of Van Lenten and Fogelman and Van der Westhuyzen et al., who found that the intralysosomal degradation of lipoproteins, especially apo B, resulted in an initial limited endoproteolytic attack at susceptible sites by cathepsin D in vitro. Our studies emphasize that these mechanisms could also be of relevance to cells of the arteriosclerotic lesion in vivo.

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