On the Pathogenesis of Atherosclerosis: Enzymatic Transformation of Human Low Density Lipoprotein to an Atherogenic Moiety

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Summary

Combined treatment with trypsin, cholesterol esterase, and neuraminidase transforms LDL, but not HDL or VLDL, to particles with properties akin to those of lipid extracted from atherosclerotic lesions. Single or double enzyme modifications, or treatment with phospholipase C, or simple vortexing are ineffective. Triple enzyme treatment disrupts the ordered and uniform structure of LDL particles, and gives rise to the formation of inhomogeneous lipid droplets 10–200 nm in diameter with a pronounced net negative charge, but lacking significant amounts of oxidized lipid. Enzymatically modified LDL (E-LDL), but not oxidatively modified LDL (ox-LDL), is endowed with potent complement-activating capacity. As previously found for lipid isolated from atherosclerotic lesions, complement activation occurs to completion via the alternative pathway and is independent of antibody. E-LDL is rapidly taken up by human macrophages to an extent exceeding the uptake of acetylated LDL (ac-LDL) or oxidatively modified LDL. After 16 h, cholesteryl oleate ester formation induced by E-LDL (50 μg/ml cholesterol) was in the range of 6–10 nmol/mg protein compared with 3–6 nmol/mg induced by an equivalent amount of acetylated LDL. At this concentration, E-LDL was essentially devoid of direct cytotoxic effects. Competition experiments indicated that uptake of E-LDL was mediated in part by ox-LDL receptor(s). Thus, ~90% of ^{125}I-ox-LDL degradation was inhibited by a 20-fold excess of unlabeled E-LDL. Uptake of ^{125}I-LDL was not inhibited by E-LDL. We hypothesize that extracellular enzymatic modification may represent an important step linking subendothelial deposition of LDL to the initiation of atherosclerosis.

There is wide consensus that subendothelial deposition of low density lipoproteins (LDL)[1] represents the starting point in atherogenesis (1–7). Accumulation of this lipoprotein is followed by emigration of blood monocytes to the affected sites, by their uptake of lipid with the development of foam cells, and by the initiation of a chronic inflammatory response that may be sustained by locally released cytokines in conjunction with immunopathological cellular events (8, 9). The detrimental processes may possibly be aggravated by local infections (10). Proliferation and death of fibroblasts, smooth muscle cells, and macrophages, together with enhanced production of extracellular matrix material, cause thickening of the arterial wall and narrowing of the vessel lumen.

Studies conducted in many laboratories have led to a veritable explosion of knowledge relating to the fundamental problem of why LDL, but not other plasma lipoproteins, should be endowed with atherogenic potential. Two intriguing questions are of central interest. First, why are blood monocytes induced to emigrate to sites of LDL deposition? Second, why is cellular uptake of deposited LDL so efficient despite the fact that macrophages express only low numbers of LDL receptors in normal individuals; and, macrophage foam cell formation also occurs in individuals lacking the LDL receptor altogether.

A major advance came with the discoveries that LDL can be modified in vitro by oxidation (11–14) or acetylation (15), or by treatment with malondialdehyde (MDA) (16) to yield molecules with potentially atherogenic properties (17,

[1] *Abbreviations used in this paper:* ac-LDL, acetylated LDL; E-LDL, enzymatically modified LDL; LCA, lesion complement activator; LDH, lactate dehydrogenase; LDL, low density lipoprotein; MDA, malondialdehyde; NHS, normal human serum; ox-LDL, oxidatively modified LDL; TBARS, thiobarbituric acid–reactive substances.

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18). Oxidatively modified LDL (ox-LDL) stimulates endothelial cells to express adhesive molecules for monocytes, thus inducing their *trans*-endothelial passage in vitro (19–21). Modified lipoproteins are recognized by scavenger receptors on macrophages and are rapidly endocytosed (15, 17). In contrast to uptake of LDL, cholesterol uptake by the scavenger receptor(s) is not subject to negative feedback regulation, so that uncontrolled endocytosis will occur (15). Transformation of macrophages to foam cells in situ has, by inference, been widely thought to derive also from uptake of oxidized or chemically modified LDL via the macrophage scavenger receptor.

Characterization of lipids present in and extracted from atherosclerotic lesions has been another area of intense research. Due to the different methods used for their isolation, the lipid preparations examined in different laboratories may be heterogeneous. However, several general conclusions are probably valid, and they raise problems regarding the general validity of the concept that oxidatively modified LDL is the major instigator of atherogenesis. First, the content of oxidized lipid or MDA-LDL in lipids extracted from the lesions is, at best, minor (22). Second, lesion-derived lipid particles consist mainly of very large droplets (23–26); this morphology thus differs fundamentally from that of LDL, or ox-LDL, or MDA-LDL. Previous studies have provided evidence that formation of these large lipidic particles occurs extracellularly at an early stage of atherogenesis through fusion of LDL particles (25). Third, lesion-derived lipid particles have an unusually high content of free cholesterol (23, 24, 26–29), and this may be the reason why they potently activate the alternative complement pathway (29). Finally, lesion-derived lipid particles are rapidly taken up by macrophages and they thus emerge as candidates alternative to oxidized or MDA-modified LDL for evoking foam cell formation (22, 30).

The discovery of a pathway other than oxidation leading to transformation of LDL to a molecule displaying properties akin to those of lesion-derived lipids might obviously help to provide a more plausible working hypothesis on the pathogenesis of atherosclerosis. We reasoned that enzymes may play an important role. Tissue proteases could degrade the lipoprotein B "shell" (31), rendering underlying lipids accessible to cholesterol esterase. Release of fatty acids would increase the relative content of free cholesterol, and neuraminidase could remove an important complement inhibitor (32) from the particles. While our studies were under way, one paper appeared showing that combined treatment of LDL with trypsin and cholesterol esterase provokes fusion of LDL particles, increases their content of free cholesterol, and that such particles adhere avidly to extracellular matrix (33). We will now show that the triple enzyme combination transforms LDL, but not high density LDL (HDL) or very low density lipoproteins (VLDL) to particles endowed with potent complement-activating properties, and that this enzymatically modified LDL (E-LDL) also rapidly induces foam cell formation in human macrophages. Uptake appears to occur in part via the scavenger receptor. E-LDL thus exhibits similar properties as lesion-derived lipid particles, and we hypothesize that it could represent a major trigger of atherosclerosis.

Materials and Methods

Native human LDL, HDL, and VLDL were isolated from pooled human plasma according to conventional procedures (34). During the preparation plasma and LDL were kept under N_2 and further protected against artificial oxidation with 500 nmol/liter EDTA. Lesion complement activator (LCA) was isolated from atherosclerotic plaques as previously described (29). The cholesterol contents of the preparations were determined enzymatically using the cholesterol monotest determination kit from Boehringer-Mannheim, Mannheim, Germany.

Radioiodination of LDL. LDL was radioiodinated using 1,3,4,6-tetrachloro3,6-diphenylglycoluril (35) (Iodogen; Sigma Chemical Co., Deisenhofen, Germany). 1 ml of an LDL solution (5 mg/ml cholesterol) in PBS was adjusted to pH 9.5 with 0.3 M borate buffer (pH 10.5) and incubated with 0.75 mCi ^{125}I (Amersham, Braunschweig, Germany) in Iodogen-coated plastic caps (25 μg Iodogen/cap) for 2 × 5 min. Labeling was terminated with 2 mM NaI and 2 mM Na_2SO_3. To remove free ^{125}I, iodinated LDL was passed over a PD-10 Sephadex G25 column (Pharmacia-LKB, Uppsala, Sweden). Incorporation of ^{125}I was determined by TCA-precipitation of labeled protein and the specific activity of labeled LDL was in the order of 200 cpm/ng cholesterol.

Chemical Modification of LDL. For acetylation, LDL solutions were diluted 1:1 with a saturated Na-acetate solution and placed on ice. 1 μl acetic anhydride/mg LDL-cholesterol was added every 15 min for 1 h under gentle shaking, followed by overnight dialysis against 150 mM NaCl, 5 mM Tris, 1 mM EDTA (15). Oxidative modification of LDL was achieved as previously described (36) with slight modifications. Briefly, LDL solutions were dialyzed at 4°C overnight against EDTA-free buffer, and then diluted with Ham's F-10 medium (Flow Laboratories, Irvine, UK) to yield final concentrations of 0.5 mg/ml cholesterol, and incubated with 50 μM $CuSO_4$ (16 h, 37°C, 5% CO_2). Using this protocol, thiobarbituric acid–reactive substances (TBARS) were found to be in the range of 60 nM/mg cholesterol. Modified LDL preparations were stored at 4°C and used within 2 wk.

Measurements of TBARS were performed as described (37). Samples (0.5 ml) were mixed with 1 ml thiobarbituric acid (TBA)-solution (0.67% TBA, 0.05 M NaOH, 1 mM EDTA), 0.3 ml TCA (50%), and incubated at 95°C for 30 min in glass tubes. The samples were cooled to room temperature, mixed with 1 ml butanol, and centrifuged for 15 min at 2,000 *g*. The upper phase was removed and TBARS were determined spectrophotometrically at 532 nm in an Ultraspec II spectrometer (Pharmacia-LKB) using 1.1.3.3.tetraethoxypropane as a standard.

Hydroperoxides in LDL preparations were determined by HPLC or iodometry (38, 39). Briefly, 100-μl samples were incubated with 1 ml color reagent (No. 14106; E. Merck AG, Darmstadt, Germany) for 60 min at room temperature in the dark. Absorbance was determined at 365 nm in an Ultraspec II spectrometer using H_2O_2 as a standard.

Enzymatic LDL Modifications. LDL solutions in PBS were adjusted to a cholesterol concentration of 5 mg/ml. To 0.1 ml LDL solution were added 0.8 μl of trypsin stock solution (Sigma Chemical Co.) (1 mg/ml) and 3 μl cholesterol-esterase (Boehringer-Mannheim) (2 mg/ml). After incubation for 2 h at 37°C in a Thermomixer (Eppendorf, Hamburg, Germany; 600 rpm), the pH of the solution was adjusted to approximately pH 5.5 by addition of 0.1 ml morpholino ethane sulfonic acid (MES) buffer (50

mM, pH 4.9), and 1.9 μl Mg ascorbate (30 mg/ml) and 20 μl neuraminidase (Behringwerke, Marburg, Germany) (1 U/ml) were added. Incubations were continued for 14 h at 37°C. Thereafter, samples were diluted to 1 ml with MEM (Sigma Chemical Co.), pH 7.4, so that final concentration of cholesterol was 0.5 mg/ml. E-LDL was stored at 4°C. E-LDL preparations that were used in the degradation experiments additionally received 10 μg/ml (final concentration) aprotinin (Sigma Chemical Co.).

Monocyte Isolation. Monocytes were isolated from citrated buffy coats, which were kindly provided by the Blood Transfusion Center of the University of Mainz. Monocytes were isolated according to Denholm and Wolber (40). Monocyte cell suspensions regularly contained 70–90% monocytes as determined by flow cytometry. They were adjusted with MEM + 10% human AB serum to densities of $1.3–1.5 \times 10^6$ cells/ml. In esterification experiments, 1-ml aliquots of cell suspensions were applied per well to 24-well cell culture plates (Nunc AS, Roskilde, Denmark). In degradation experiments, 96-well plates (Nunc AS) were used and each well was seeded with 0.2 ml of the cell suspensions. The cells were cultured for 7 d at 37°C, 5% CO_2, and medium, supplemented with 10% AB serum, was renewed every 2 d.

Determinations of Cholesteryl Oleate Formation in Macrophages. 0.1 ml of a 500-nM [^{3}H]oleic acid solution with an activity of 2.2×10^7 cpm/ml was added together with 0.9 ml of fresh MEM medium (without serum) containing the respective LDL preparation to cells, and incubations were performed at 37°C, 5% CO_2. Thereafter, the cells were washed twice with 0.5 ml MEM. Lipids were extracted by 30-min incubations with a 3:2 *N*-hexane-isopropanol solution, followed by two further extractions with this solvent. Lipids were dried under vacuum, taken up in a 2:1 (vol/vol) chloroform/methanol solution, and applied to TLC plates (Silicagel-TLC-DC-plates; Machery & Nagel, Düren, Germany). Chromatographies were performed in hexan-diethyl ether and acetic acid (83:16:1) and lipid standards (Sigma Chemical Co.) were run in parallel. The lipid standards were reacted with 8-anilin-1-naphtalene-sulfonic acid solution (Sigma Chemical Co.) and visualized under a UV lamp. The cholesteryl oleate band was retrieved, admixed with 10 ml scintillator, and radioactivity determined in a beta counter (LS 6000 TA; Beckman Instruments, Frankfurt, Germany). Calibrations were performed by chromatography of 50 μl and 100 μl of the oleic acid stock solution. Cholesteryl oleate formation was expressed in picomoles oleate per milligram cell protein (41).

For determination of cell protein, samples were incubated with 0.5 ml of 0.2 N NaOH. Protein determinations were performed using the Bradford reagent (Bio-Rad Laboratories, Munich, Germany) with human albumin as a standard. Direct fluorescent staining of intracellular lipids was performed by incubating cells with 10 μM Nile red solution as described (42).

Degradation Assays. Degradation assays were performed essentially as described previously (12, 43). In brief, after 5 h of incubation of cells with the respective LDL preparations, cells were chilled on ice and media were removed. The plates were then washed and cellular ATP was measured. ^{125}I-labeled degradation products were determined in the supernatants (0.2 ml samples) by adding 10 μl buffer containing 20% BSA and 0.1 ml of 20% TCA. After centrifugation, 0.1 ml silver nitrate (5%) was added to the supernatant to precipitate free ^{125}I. Noniodine, TCA-soluble radioactivity in the supernatant was then determined in a Cobra Auto Counter (model 5005; Packard Instrument Co., Meriden, CT). To correct for degradation products produced during the preparation, control incubations were performed in cell-free plates and the values were subtracted from the experimental values.

Other Determinations. Cellular ATP levels were determined using a commercial test kit (No. 567736; Boehringer-Mannheim) according to the manufacturer's protocol. Briefly, the cells were lysed with 1% Triton X-100, mixed with the luciferase reagent, and bioluminescence was determined in a Biolumat LB 9500 (Berthold Laboratories, Wildbad, Germany).

LDH release into the culture medium was measured as follows. 50 μl of cell supernatant was mixed with 1 ml of a test buffer containing 0.2 mM NADH, 5 mM EDTA, 50 mM triethanolamine-HCl, pH 7.6. Reaction was started by addition of pyruvate to 2 mM. Lactate dehydrogenase (LDH) activity was determined as the decrease of OD_{366}/10 min. Maximum release was determined in medium of cells lysed with 0.1% Triton X-100. Spontaneous release from untreated cells was always <3% of maximal release.

Determination of Complement Activation. Normal human serum was prepared from freshly drawn blood of healthy individuals. Blood was allowed to clot for 30 min at 4°C and subsequently centrifuged at 2,000 *g* for 10 min. Serum was used either directly or given 10 mM EGTA, 2 mM $MgCl_2$, to inhibit classical pathway activation. Inhibition of the classical pathway by EGTA was ascertained by demonstrating total absence of hemolytic activity of EGTA-normal human serum (NHS) on antibody-laden sheep erythrocytes. Preservation of alternative pathway activity was ascertained by showing that EGTA-NHS could still lyse rabbit erythrocytes. NHS or EGTA-NHS was incubated with 1 vol lipoprotein solution and incubated at 37°C in a thermomixer under continuous shaking (500 rpm/min) for 2 h. Reactions were stopped by adding 10 mM EDTA. For maximum C3 conversion and maximum generation of SC5b-9, serum samples diluted with 1 vol of MEM were activated with particulate inulin (Merck) for 2 h at 37°C. Inulin particles were subsequently removed by centrifugation.

Conversion of C3 was assessed by two-dimensional immunoelectrophoresis as described (44). Briefly, 6 μl of samples was applied to 1% agarose gels in 0.1 M glycine, 0.038 M Tris, pH 8.7, and albumin stained with bromphenol blue was allowed to migrate 4–5 cm (applied voltage: 10 V/cm). Second dimension immunoelectrophoresis was performed using anti-C3c (Dakopatts Immunoglobulins, Copenhagen, Denmark) incorporated into the gel at a final concentration of 1.2 μl/cm^2. Second-dimension immunoelectrophoresis was performed overnight at 2 V/cm at room temperature. Gel plates were pressed, washed, and stained with Coomassie blue according to conventional procedures.

Formation of terminal complement complexes SC5b-9 was determined by ELISA as previously described with the following modifications (45). Instead of the biotinylated anti–rabbit Ig from donkey (Amersham International, Little Chalfont, UK) and the streptavidin-biotinylated peroxidase complex (Amersham International), a peroxidase-conjugated anti–rabbit Ig from swine (Dakopatts Immunoglobulins) diluted 1:1,000 was applied as the revealing antibody. Absorbance was read at 492 nm in an Easy Reader (EAR 400; SLT Instruments, Grailsheim, Germany).

Agarose Gel Electrophoresis of LDL. Agarose gel electrophoresis was performed using a commercially available kit (Lipidophor; Immuno AG, Vienna, Austria). Immune precipitation of apolipoprotein B-100 was done with an anti–apo B rabbit anti–human antibody (Beckman Instruments, Irvine, CA) essentially as described by Armstrong et al. (46). Gels were dried and immune precipitates were stained using Coomassie blue. Coomassie brilliant blue R was obtained from Sigma Chemical Co., St. Louis, MO, and was dissolved (0.2% wt/vol) in 30% isopropanol/10% acetic acid/60% water. Slides were stained for 15 min followed by destaining of the background using the solvent mentioned

above lacking the Coomassie stain. After washing thoroughly with water, slides were dried at room temperature.

Quantitative rocket immunoelectrophoresis was performed as described previously (29). Polyclonal rabbit antibodies against apo B used in these experiments were obtained from Dakopatts Immunoglobulins.

Electron Microscopy. Preparations of native and E-LDL were applied as drops to electron microscope grids carrying thin carbon-coated Formvar films, made hydrophilic by glow discharge shortly before use. Adsorbed particles were negatively stained with 2% sodium silicotungstate as described (47). The specimens were examined in a electron microscope (100 CX; JEOL, Stockholm, Sweden) equipped with a high resolution objective lens pole piece (spherical abberation coefficient, C_s 0.7 mm) and operated at 80 kV. Images were recorded by a low dose exposure technique on Agfa Scientia 23D56 plates (Agfa, Leunkusen, Germany) developed to an electron speed of 0.7 $\mu m^2 e^-$. Magnification was calibrated using a Fullam SiO_2 grating replica No. 6002 (Fullam Inc., Schenectady, NY).

Results

Enzymatic Transformation of LDL to a Complement Activator. The capacity to activate the alternative complement pathway was a remarkable feature of lipid isolated from atherosclerotic lesions (29). It seemed to us probable that the LCA was derived from plasma lipoproteins, so we commenced our present investigations by attempting to generate a complement-activating lipid from these moieties. All attempts to enzymatically transform HDL and VLDL to complement activators failed. In contrast, LDL acquired complement-activating capacity after sequential treatment with trypsin, cholesterol esterase, and neuraminidase. Assays for C3 conversion and SC5b-9 formation were conducted in 50% NHS and in 50% NHS + 10 mM EGTA/2 mM $MgCl_2$. Similar results were obtained in both systems. The classical pathway was ascertained to be totally inhibited in EGTA/$MgCl_2$, and the results thus showed activation to proceed via the alternative pathway. A panel of two-dimensional immunoelectrophoreses showing C3 conversions in 50% NHS is depicted in Fig. 1. Inulin-activated serum was run as a positive, and the enzyme cocktail incubated with serum was included as a negative control. The effects of ox-LDL or native LDL on C3-turnover were investigated in parallel. Incubation of serum with neither the enzyme cocktail nor with ox-LDL or LDL led to significant C3 conversion. Maximal C3 conversion occurred in inulin-activated serum. When E-LDL was added, marked C3-conversion was always observed. By planimetry, we estimated that E-LDL added at a final concentration of 0.4 mg cholesterol/ml to 50% serum provoked over 75% of C3 conversion in 2 h at 37°C. These results were obtained without exception with 10 different E-LDL preparations. Complement activation also occurred in serum that was depleted by >95% of IgG by a passage over protein G–Sepharose at 0°C. Hence, activation occurred via the alternative pathway and was not due to formation of immune complexes. Marked C3 conversion was never observed if LDL was modified with a single or any double enzyme combination.

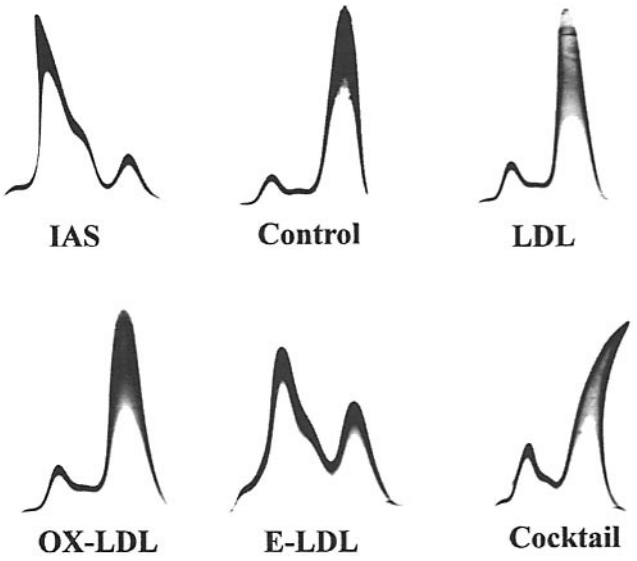

Figure 1. Analyses of C3-conversion in human serum by crossed immunoelectrophoresis. An equivalent of 3 μl serum was electrophoresed in each plate. First-dimension electrophoresis was from right to left; the agarose plates contained 1.2 $\mu l/cm^2$ of polyclonal rabbit antibodies against C3. Control serum exhibited the typical pattern, with a major native C3 precipitate and only small amounts of C3 cleavage products. Addition of native LDL or ox-LDL or the enzyme cocktail to serum did not induce any marked alteration in the electrophoresis pattern. Addition of inulin (inulin-activated serum: *IAS*) led to almost complete conversion of C3; addition of E-LDL also led to marked conversion of C3.

The complement cascade does not inevitably proceed beyond the stage of C3 conversion. Cleavage of C5 is biologically of central importance because this generates the chemoattractant C5a. We therefore assessed activation of the terminal complement sequence by quantifying SC5b-9 complexes. Fig. 2 *A* shows the results of experiments conducted with NHS and EGTA-NHS spiked with E-LDL or LDL at 0.4 mg cholesterol/ml. Serum activated with inulin and containing maximal levels of SC5b-9 (~200 μg/ml) served as positive control. Native LDL or LDL modified with single or double enzyme treatments, or the enzyme cocktail alone provoked little generation of SC5b-9. However, LDL treated with the triple enzyme combination was an effective generator of SC5b-9 in the presence or absence of EGTA. ox-LDL was ineffective (not shown).

In a further experiment, E-LDL was added at increasing final concentrations of cholesterol to NHS, and the dose-response curve of SC5b-9 formation shown in Fig. 2 *B* was obtained. Significant activation of the terminal sequence was detected at E-LDL cholesterol concentrations of ≥200 μg/ml. This dose-response was comparable to that previously observed with cholesterol lipid isolated from atherosclerotic lesions (29).

Properties of Enzymatically Modified LDL. E-LDL contained no oxidized lipids, as revealed by determination of lipid hydroperoxides and TBARS. The concentration of hydroperoxides was below the level of detection in samples of E-LDL and the enzyme cocktail. One preparation of

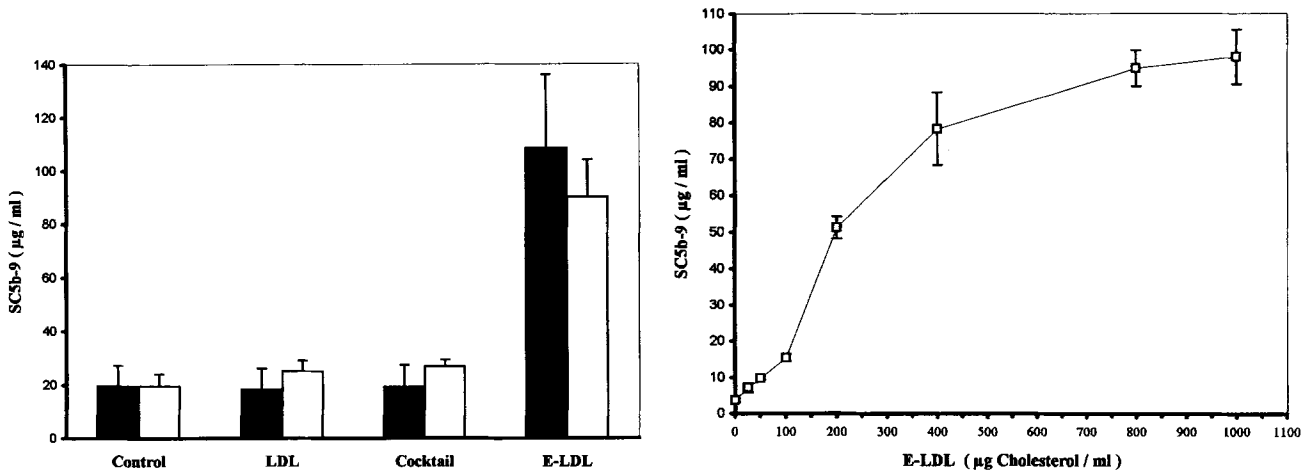

Figure 2. (*A*) Determination of SC5b-9 in human serum by ELISA. Serum samples were incubated with buffer (*control*), LDL, the enzyme cocktail, or E-LDL in the presence (*open bars*) or absence (*closed bars*) of 10 mM EGTA/2 mM $MgCl_2$ for 2 h, 37°C. The final concentration of LDL and E-LDL added was 0.4 mg/ml cholesterol. Only E-LDL generated large amounts of SC5b-9, and activation occurred via the alternative complement pathway (n = 3, ±SD). (*B*) Dose-response curve of SC5b-9 generation in human serum induced by E-LDL. E-LDL was added to human serum at the given final concentrations of cholesterol and SC5b-9 was measured after 2 h, 37°C. Significant SC5b-9 generation was observed at 200 µg cholesterol/ml of E-LDL (n = 3, ±SD).

ox-LDL used as a positive control had a hydroperoxide concentration of 25 nmol/mg cholesterol in a parallel determination. Determination of TBARS with the spectrophotometric method yielded low absorbances with E-LDL that were identical with background values obtained with the enzyme cocktail. With the same method, TBARS of 60 nmol/mg cholesterol were measured in a preparation of oxidized LDL. Furthermore, no conjugated dienes could be detected in E-LDL preparations using HPLC. In this regard, E-LDL thus also appeared similar to LCA. The relative charge density of E-LDL was assessed in comparison to LDL and ox-LDL by agarose gel electrophoresis. As shown

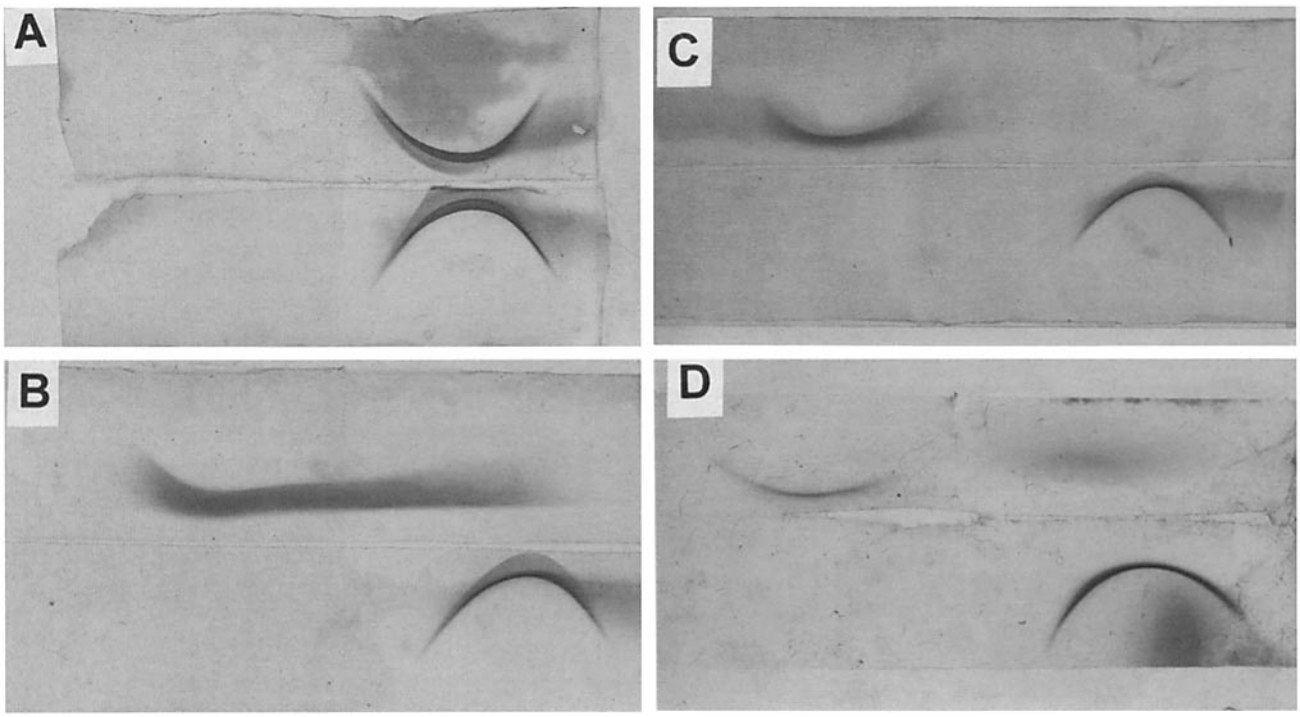

Figure 3. Immunoelectrophoresis of LDL, E-LDL, ac-LDL, and ox-LDL developed with antibodies against apolipoprotein B. First-dimension electrophoresis was from right to left, and native LDL was applied as control in each plate (lower precipitate of each pair). (*A*) LDL after 48 h incubation in buffer, 37°C; (*B*) $CuSO_4$-oxidized LDL; (*C*) acetylated LDL; (*D*) E-LDL. E-LDL was observed to exhibit a strong net negative charge similar to ac-LDL and ox-LDL. The weaker precipitate generated by E-LDL was interpreted to reflect loss of epitopes in the molecule.

by immune electrophoresis (Fig. 3), E-LDL exhibited a strong negative charge similar to acetylated LDL (ac-LDL) and ox-LDL. The total amount of antigen applied in each analysis was identical, and the weaker precipitate observed with E-LDL indicated an overall reduction in epitopes. In fact, prolonged degradation (3–7 d) with the enzyme cocktail caused total disappearance of the precipitate (not shown). In our previous work, we also found that LCA was not precipitable with antibodies against apo B (29).

ox-LDL has been reported to exert direct cytotoxic effects (48), and it was important for our further studies to determine whether E-LDL was also cytotoxic. Macrophages were incubated with E-LDL, and LDH release to the supernatant as well as cellular ATP levels were measured after 5 and 16 h. It was found that E-LDL exerted little direct cytotoxic effects when applied at $\leq$50 μg/ml cholesterol. Some cytotoxicity was noted at higher concentrations. We also observed that cytotoxicity increased if E-LDL was stored for days at 4°C. The cause for this was not investigated, but stored E-LDL was not used in these degradation experiments (see below) where cytotoxicity might have generated artifacts.

By electron microscopy, we observed that single enzyme treatments induced no significant change in LDL micromorphology. Treatment with phospholipase C or simple vortexing were also without gross effect. Treatment with trypsin + cholesterol esterase did provoke some fusion of LDL particles. Treatment with a triple enzyme combination always provoked complete disruption of the uniform LDL structure and reorganization of lipids into inhomogeneous droplets of 10–200 nm in diameter (Fig. 4). The morphology of these droplets showed clear resemblance to that of the LCA described previously (29), and to that of lipid droplets isolated in other laboratories, particularly by Kruth et al. (49). Their dimensions also corresponded to lipid partices detected in situ by deep-freeze etch electron microscopy (25).

Rapid Uptake of Enzymatically Modified LDL and LCA by Human Macrophages. The next experiments were undertaken to determine whether E-LDL and LCA would in-

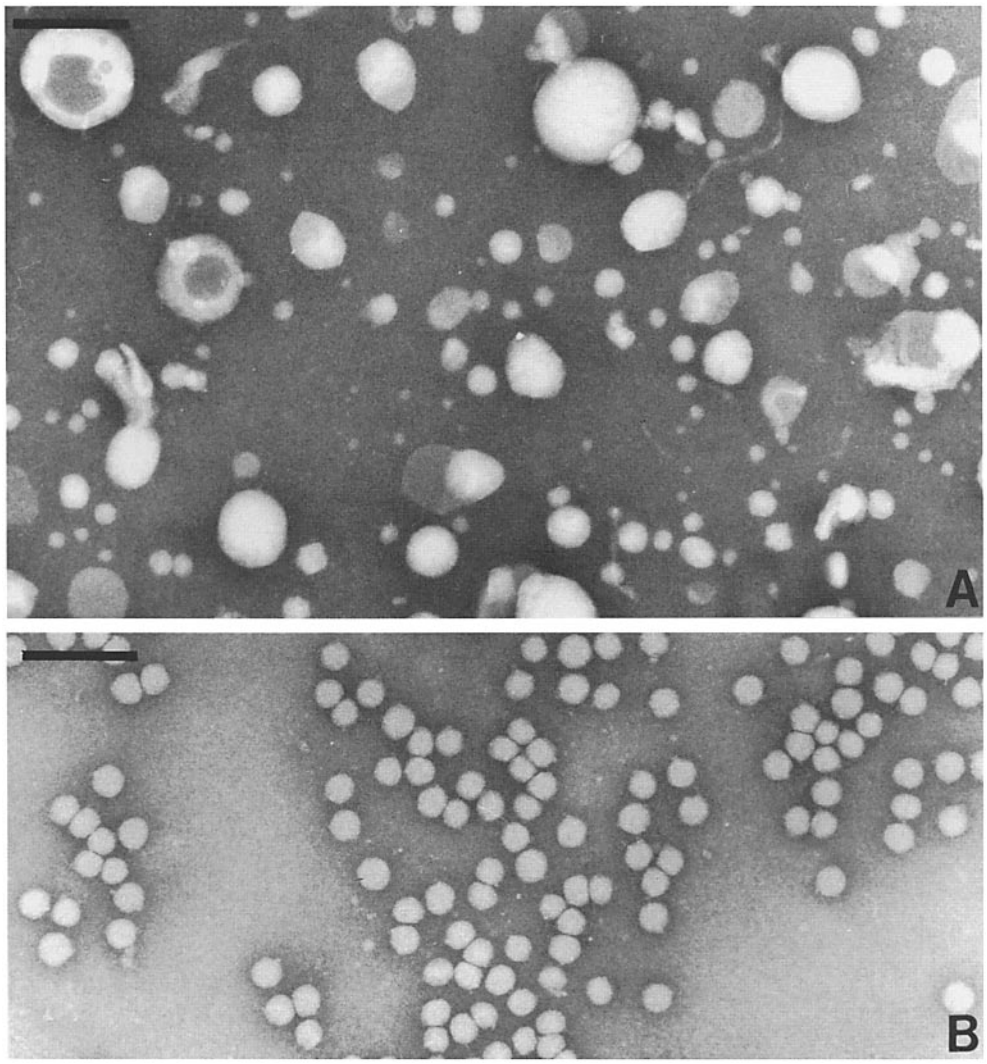

Figure 4. Electron micrographs of negatively stained preparations of E-LDL (*A*) and native LDL (*B*). The triple enzyme treatment caused disruption of the uniform LDL particle structure and led to formation of an inhomogenous population of lipid particles ranging from 10 to 200 nm in diameter (the largest particles not shown in this micrograph). A number of the particles exhibited a central stain deposit indicating a hollow core. Bars, 100 nm.

duce foam cell formation in human macrophages. As a measure for cholesterol uptake, we quantified the formation of cholesteryl oleate as described by Brown and Goldstein (41). When freshly isolated monocytes were incubated with E-LDL or LCA, enhanced formation of cholesteryl oleate was noted, but absolute values of cholesteryl ester formation were low. Typically, overnight incubation of monocytes with LDL and E-LDL with 50 μg/ml cholesterol resulted in formation of 200–400 pmol cholesteryl oleate/mg protein and 600–800 pmol cholesteryl oleate/mg protein, respectively; cholesteryl oleate formation induced by LCA was in the same range as found for E-LDL (data not shown in detail). Incubation with ox-LDL or ac-LDL resulted in formation of 800–1,000 pmol cholesteryl oleate/mg protein. The situation was different when cultured human macrophages were used. In these cases, we noted a dramatic uptake of E-LDL (n = 10) or LCA (n = 3). LDL treated with single or double enzyme combinations did not markedly promote cholesteryl oleate formation. When offered to macrophages at 100 μg/ml cholesterol, LDL provoked formation of <600 pmol cholesteryl oleate/mg protein, ac-LDL and LCA induced 3,000–6,000 pmol cholesteryl oleate/mg protein, and incubation with E-LDL led to formation of >6,000 pmol cholesteryl oleate/mg protein. Fig. 5 *A* depicts the collective results of six independent experiments with E-LDL and two experiments with LCA, wherein the lipoproteins were incubated at 100 μg/ml cholesterol with human macrophages for 16 h, and cholesteryl oleate formation was expressed as a percentage of that observed with ac-LDL. The latter was set at 100% for each set of determinations. It can be seen that E-LDL was even more effective in promoting cholesteryl oleate formation than ac-LDL. Values found for ox-LDL were in turn always lower than for ac-LDL (not shown). A dose-response curve for cholesteryl oleate formation induced by E-LDL is shown in Fig. 5 *B*. Cholesteryl oleate formation virtually plateaued at cholesterol concentrations of 25–50 μg/ml. An essentially similar dose-response pattern was originally found by Goldstein et al. in their studies of cholesterol uptake via ac-LDL in mouse macrophages (15).

The results of an experiment to assess the kinetics of cholesteryl oleate formation are shown in Fig. 6. Cholesteryl oleate formation provoked by E-LDL or LCA (not shown) displayed essentially the same kinetics as observed with acetylated LDL or ox-LDL, commencing at ~2 h and continuing over 16 h. These results with ac-LDL and human macrophages shown here are similar to those originally reported for mouse macrophages.

In additional experiments intracellular lipids were stained with Nile red (42) and cells examined by fluorescence microscopy. Foam cell formation by E-LDL was directly evident (Fig. 7).

To obtain an indication whether uptake of E-LDL occurred via a scavenger receptor–dependent pathway, competition experiments were performed in which LDL degradation was measured. We used radioiodinated, ox-LDL, ac-LDL, or native LDL as ligands and measured apo B degradation in the presence or absence of unlabeled competitors. E-LDL was freshly prepared to avoid cytotoxic effects. This protocol yielded reproducible results, whereas employment of radioiodinated E-LDL was impossible because of unacceptably high backgrounds generated by enzyme treatment of LDL.

As shown in Fig. 8 *A*, E-LDL, but not native LDL, effectively inhibited degradation of ^{125}I-ox-LDL. Control experiments using the homologous ligand showed 90% inhibition by 50 μg cholesterol/ml ox-LDL, when 5 μg cholesterol/ml of ^{125}I-ox-LDL was offered as substrate. E-LDL also clearly inhibited ox-LDL uptake and degradation, albeit to a less effective extent. 25 μg cholesterol/ml E-LDL inhibited degradation of ^{125}I-ox-LDL by ~35%, and 50 μg/ml E-LDL effected ~75% inhibition. Since the

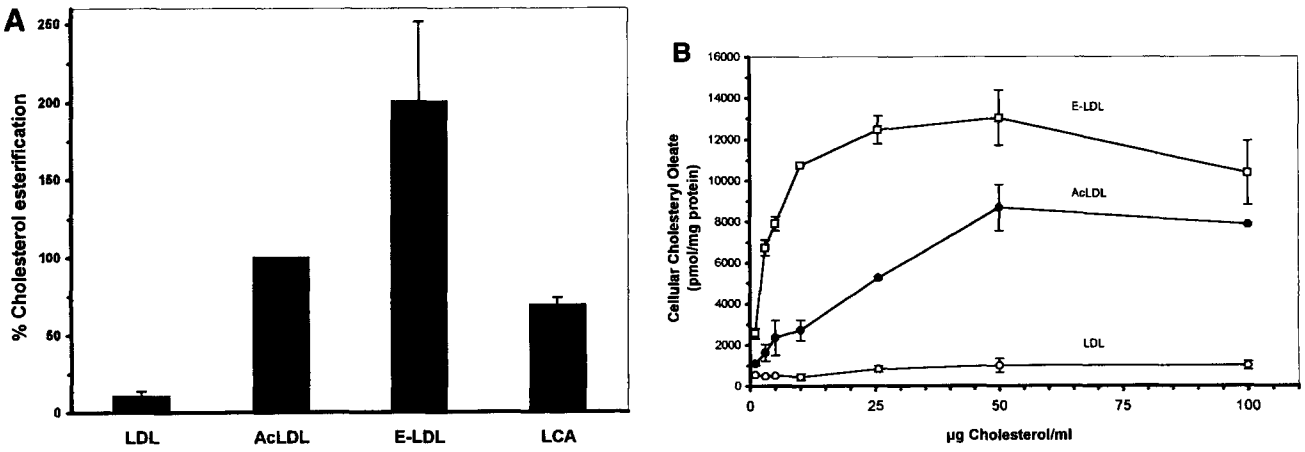

Figure 5. *(A)* Measurements of cholesteryl-oleate formation in human macrophages. Cells were incubated with LDL, ac-LDL, E-LDL, or LCA at a final cholesterol concentration of 50 μg/ml for 16 h in the presence of [^{3}H]oleate. Thereafter, cell-associated cholesteryl oleate was quantified. The amount of cholesteryl oleate determined in cells that had been incubated with ac-LDL was set at 100%, and the relative amounts of cholesteryl oleate found in the other samples were related to this value. Both LCA (n = 3) and E-LDL (n = 10) induced marked cholesteryl oleate formation (values are shown ±SD). *(B)* Dose-response curve of cholesteryl oleate formation in human macrophages induced by E-LDL, ac-LDL, and LDL (n = 4, ±SD).

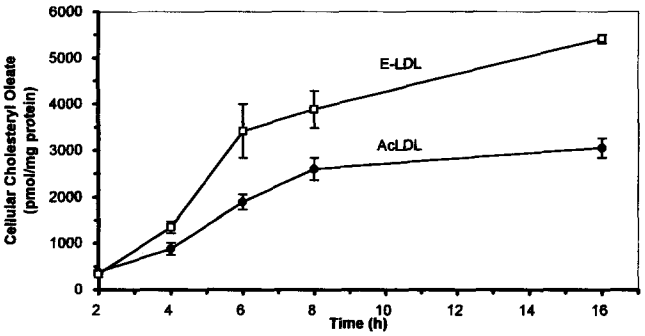

Figure 6. Kinetics of cholesteryl oleate formation induced in human macrophages by ac-LDL and E-LDL, each added at final cholesterol concentration of 10 μg/ml. Values are mean ±SD of duplicate determinations of a representative experiment.

latter concentration was essentially devoid of direct cytotoxic effects, the observed inhibition was considered a consequence of competition by E-LDL for the scavenger receptor. The reduced efficiency of E-LDL to inhibit ox-LDL uptake may partly have been due to the altered physical properties resulting from aggregation and fusion of the enzymatically altered LDL particles.

When either ^{125}I-ac-LDL or ^{125}I-LDL was offered as ligand (5 μg cholesterol/ml), it was found that E-LDL did not significantly inhibit degradation (Fig. 8, *B* and *C*), whereas competition with the respective homologous ligand occurred as expected. These findings were interpreted to indicate that E-LDL uptake occurred at least partially via a scavenger receptor pathway that was specific for ox-LDL.

Discussion

The results of this study show that human LDL, but not HDL or VLDL, can be altered by treatment with an enzyme combination to yield a lipid with atherogenic potential and with properties akin to those previously reported for lipids extracted from atherosclerotic lesions. The enzymes required for effecting this transition are widely distributed in microsomal cellular compartments. Similar to all other investigations reporting methods for creating fused LDL particles in vitro, the present work does not provide direct evidence for the presence of the modifying agents in vessel walls. This task is fraught with difficulties and we are therefore confined to making a hypothesis at the present stage. Should enzymatic modification of LDL occur in the

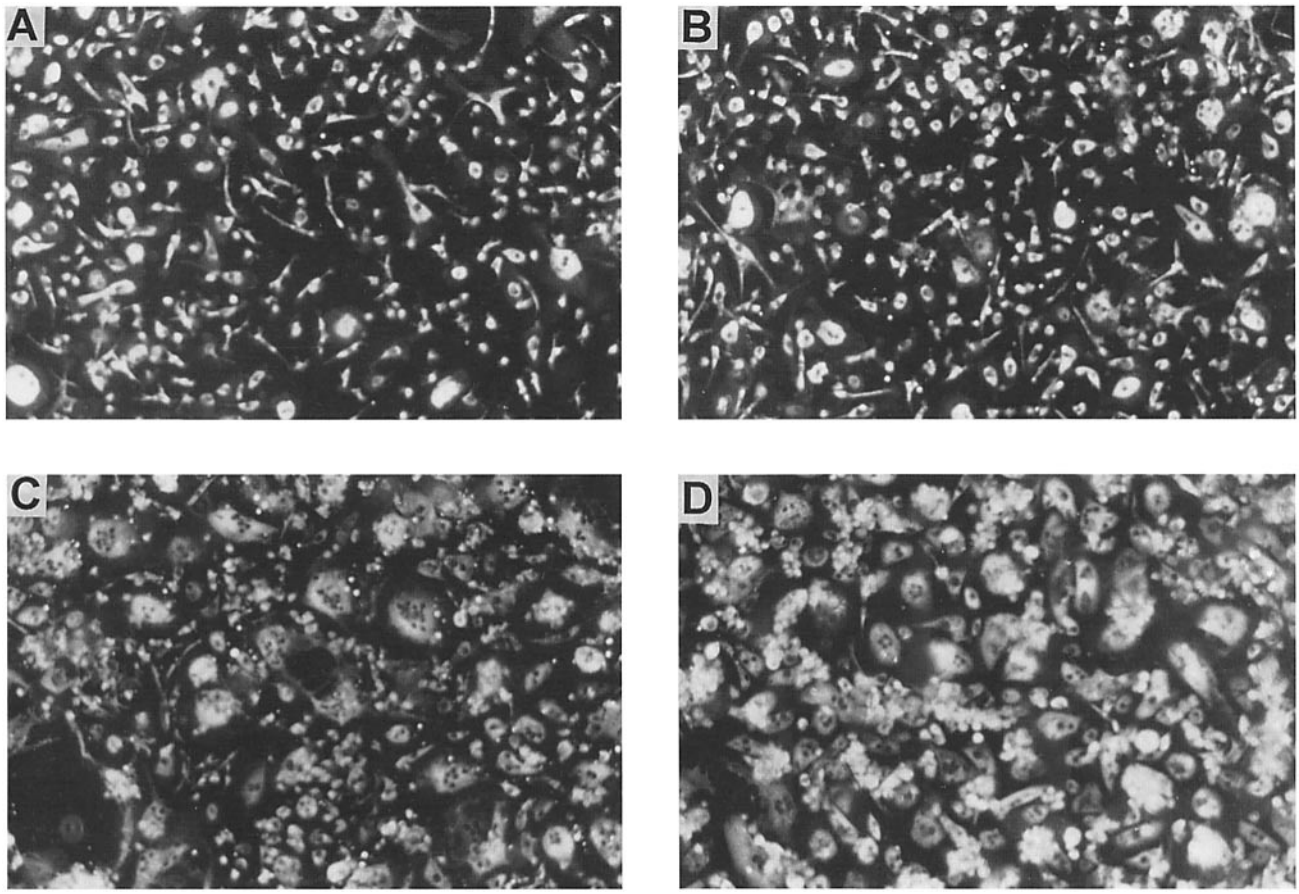

Figure 7. Fluorescent staining of intracellular lipid with Nile red. Human macrophages were incubated with buffer (*A*), LDL (*B*), ac-LDL (*C*), or E-LDL (*D*) at a final cholesterol concentration of 50 μg/ml for 16 h. Staining with Nile red revealed the marked accumulation of intracellular lipid in cells that had been incubated with ac-LDL and E-LDL (×100).

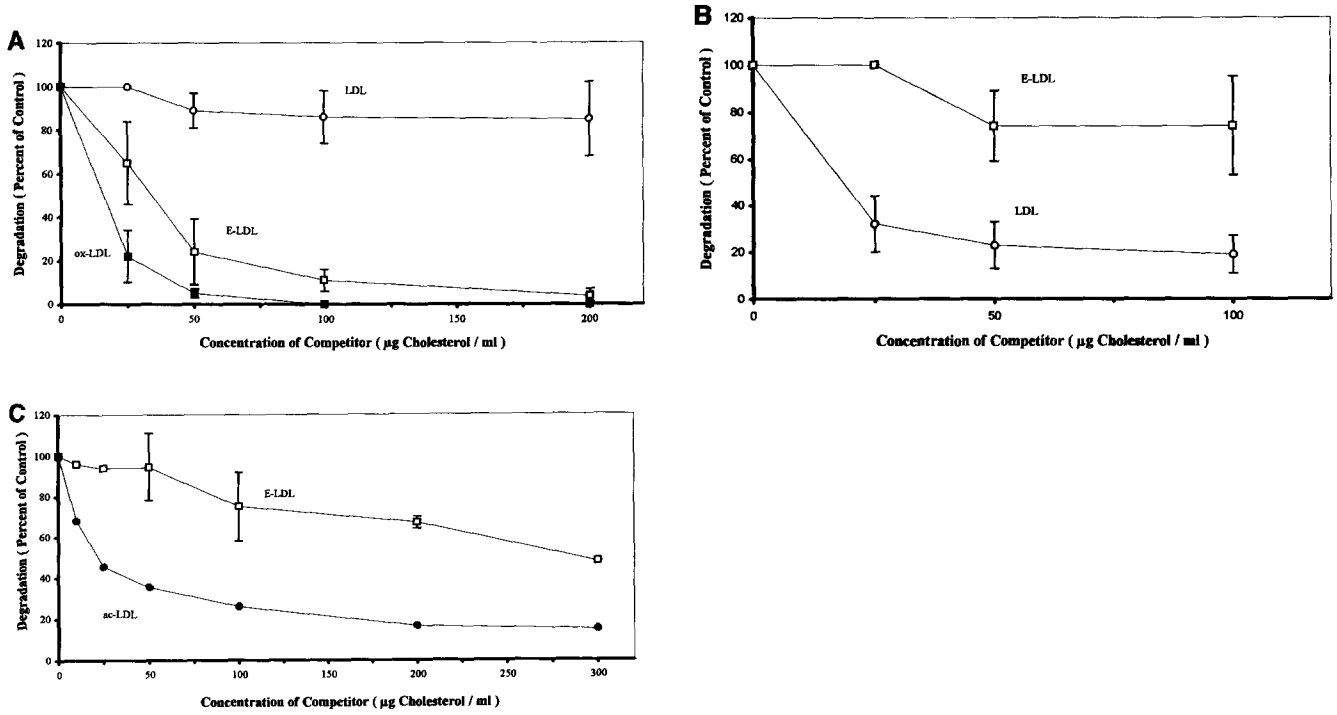

Figure 8. Evidence that E-LDL uptake is mediated at least in part via scavenger receptors. (*A*) Human macrophages were incubated with ^{125}I-ox-LDL (5 μg cholesterol/ml) in the presence of the depicted cold competitors, and degradation of the radiolabeled ligand was determined after 5 h. Ox-LDL and E-LDL inhibited degradation of radioiodinated ox-LDL, whereas LDL did not (n = 3, ± SD). (*B*) In the converse experiment, radioiodinated LDL (5 μg cholesterol/ml) was offered as ligand. In this case, the degradation of LDL could not be significantly inhibited through the addition of E-LDL (n = 3, ±SD). (*C*) When ^{125}I-ac-LDL was used as the ligand, E-LDL also did not effectively inhibit uptake and degradation (n = 2, ±SD).

subendothelium, a simple concept for the initiation of atherosclerosis emerges. Deposition and subendothelial accumulation of LDL is the first and essential step. This is aggravated by high plasma LDL levels combined with various factors that promote transendothelial insudation of the lipoprotein (50). Oxidation of LDL may play a contributing role, since ox-LDL could locally activate and damage the endothelium, promoting lipoprotein extravasation and release of microsomal enzymes (17, 48). In the subendothelium, apo B is degraded by proteases; this is known to render cholesterol esters accessible to the action of cholesterol esterase (33). The double enzyme modification promotes first fusions of LDL particles. These particles adhere avidly to extracellular matrix (33), and their fate to remain entrapped in the vessel wall is thus sealed. As sialic acid is removed, further fusions occur and the particles acquire the capacity to activate the alternative complement pathway. Activation occurs to completion with the generation of C5a and is probably due to the high content of free cholesterol in the particles. Activation is independent of IgG or formation of immune complexes. C5a that is generated may represent an important chemotactic stimulus serving to attract monocytes to the lesions. These cells differentiate within days to macrophages expressing high numbers of scavenger receptors. Uptake of E-LDL occurs at least in part via the scavenger receptor pathway, evoking foam cell formation and triggering cellular reactions that together propagate progression of the inflammatory lesion. As is evident from the reported experiments, these processes would be expected to occur in tissues in the presence of the modifying enzymes. It may be stressed that all experiments described herein were performed with human LDL and human macrophages.

The hypothesis detracts attention away from the hitherto dominating concept of oxidative LDL modification being the key process triggering atherogenesis. We do not contend that oxidation is irrelevant; but propose that enzymatic modification may be yet more widespread and possibly represent an essential feature in the pathogenetic process. As demonstrated by Steinbrecher and Longheed (22), lipids extracted from atherosclerotic lesions contain little oxidized fatty acids or MDA-modified LDL. LDL modified enzymatically in vitro also contains little oxidized lipids; thus, oxidation is not essential for transformation of LDL to an atherogenic particle. E-LDL possesses one potentially important property that ox-LDL does not, i.e., the capacity to spontaneously activate complement and generate C5a. This is in itself a remarkable finding, and it is the first and only known example for the transformation of a plasma component to a complement activator. Of note, neither HDL nor VLDL exhibit this capacity.

Three important points warrant discussion here. First, E-LDL particles fuse to form large lipidic particles. Beautiful ultrastructural studies by Frank and Fogelman have pro-

vided persuasive evidence that subendothelially deposited LDL particles actually do fuse together at an early stage of atherosclerosis; this process occurs extracellularly and precedes the influx of monocytes and foam cell formation (25). In their experiments, rabbits were fed a cholesterol-rich diet and first subendothelial deposition and fusion of LDL particles were observed after 10 d. In vitro, neither oxidative modification nor modification with MDA provokes similar fusion of LDL particles. Therefore, enzymatic modification emerges as the most probable candidate for effecting this morphological transition in vivo. Attention is here drawn to several earlier studies that described the presence of such abnormally large lipid droplets in atherosclerotic lesions. The first reports came from the work of Kruth et al. in 1984 (23, 24), and were followed by work of Simionescu (26, 51). In 1990, we purified large lipidic particles from atherosclerotic plaques and demonstrated their capacity to activate the alternative pathway (29). In 1992, Steinbrecher and Longheed showed that LDL-derived, large lipid particles isolated from plaques provoked foam cell formation (22).

The second point relates to the complement-activating capacity of E-LDL. First studies on the presence of activated complement in atherosclerotic lesions appeared in 1985 (52), yet the possible participation of complement in the pathogenesis of atherosclerosis has remained largely ignored. In 1990, we isolated terminal C5b-9 complexes from human atherosclerotic plaques and thus provided conclusive evidence that complement activation does occur in the lesions. That formation of the lipidic LCA occurs extracellularly rather than intracellularly (as a secondary step after lipid ingestion by macrophages) was originally deduced from the finding that complement deposition occurred in animal experiments at a very early stage, paralleling lipid accumulation but clearly preceding infiltration by monocytes (53). Our present studies show that LDL can be rapidly transformed to a complement activator in vitro in the absence of cells and thus strengthens this contention. It is further noteworthy that the described enzymatic treatment also markedly altered the precipitating properties of LDL; prolonged treatment led to loss of precipitability. In this regard also, E-LDL thus behaves similarly to LCA, which was previously found not to be precipitated by antibodies against apo B (29). The discovery that enzymatic modification of LDL creates large lipid particles with properties that are essentially identical to those previously found for lipid droplets isolated from atherosclerotic plaques now provides a most satisfying explanation for several intriguing and hithertofore poorly understood findings in the literature. Complement activation in the early stages of atherogenesis would inevitably trigger a variety of biologically relevant processes. Generation of anaphylatoxins is known to provoke inflammation and to attract phagocytes to the afflicted sites. Covalent attachment of C3b to activator particles leads to their opsonization and phagocytic uptake. In this regard, it will be of interest to learn whether uptake of E-LDL by macrophages is accelerated or enhanced in the presence of complement. Another aspect relates to the possible biological significance of C5b-9 formation. If any attack on innocent bystander cells occurs, this would trigger an array of secondary reactions that could further contribute to the progression of the lesion (54).

The third point relates to the capacity of E-LDL to be rapidly taken up by macrophages and to transform these to foam cells. We have found that LCA is also endowed with this property. In this regard, E-LDL is even more effective than ox-LDL or ac-LDL. Mechanisms of E-LDL and LCA uptake remain to be fully elucidated, and it is realized that the data presented in this first communication are preliminary. Our attempts to more completely delineate the uptake mechanisms have been confounded by difficulties in preparing satisfactory radioiodinated E-LDL (or LCA) tracers. E-LDL and LCA tend to aggregate and adhere nonspecifically to plastic and glass, so that we have not yet been able to conduct binding studies. Because of particle aggregation, which apparently enhances spontaneous cytotoxicity, we have also not yet undertaken to isolate subpopulations of E-LDL. These shortcomings notwithstanding, we do have cause to believe that E-LDL uptake proceeds in part via a scavenger receptor–dependent pathway. This conclusion is based on the finding that E-LDL applied at noncytotoxic concentrations inhibited degradation of ox-LDL, albeit with somewhat lowered efficiency compared with ox-LDL itself (Fig. 8). In these experiments, oxidatively modified LDL was offered as the radiolabeled ligand. In contrast, E-LDL did not significantly inhibit uptake and degradation of ^{125}I-ac-LDL. These findings can be accommodated within the framework of current knowledge on scavenger receptors. Thus, there exist at least three different classes of scavenger receptors (55, 56). The first class, termed class A by Krieger and Herz (55), binds both ac-LDL and ox-LDL, and is inhibitable by polyanions. The second class (class B) comprises CD36 and related molecules. CD36 is present in macrophages and mediates uptake of ox-LDL that is not inhibitable by ac-LDL (55, 57). The existence of this class of receptors was first reported by Sparrow et al. in 1989 (58), and the identification of CD36 followed in 1993 by Endemann et al. (57). Polyanions do not inhibit uptake of lipoproteins by this class of receptors (55, 56). In fact, we found that fucoidan did not inhibit uptake of E-LDL and in this regard, our data may be related to results of Steinbrecher and Longheed, who made the same observation with lipids that they had isolated from atherosclerotic lesions (22). Thus, CD36 or a related class B scavenger receptor emerges as a possible candidate that mediates uptake of E-LDL. Further studies are required, however, especially since a third group of perhaps heterogeneous molecules may also fulfill scavenger receptor functions (55, 56). At this stage, we certainly cannot exclude the possibility that E-LDL uptake may occur by a pathway additional to that mediated by known scavenger receptors.

A few other papers have recently dealt with in vitro extracellular modifications of LDL. Guyton et al. reported that simple vortexing provoked LDL aggregation (59). Other properties of vortexed LDL were not mentioned, but we have not found that such preparations activate com-

plement or induce foam cell formation. Suits et al. described the aggregation of LDL particles after treatment with phospholipase C (60). We detected neither complement activation nor promotion of cholesterol esterification by such preparations. The modification most closely approaching our protocol was the combined trypsin/cholesterol esterase treatment described by Chao et al. (33). When enzymatic treatment was performed as described by these authors, we found LDL aggregation to be minimal; the modified LDL preparations were not endowed with marked complement activation capacity and also did not induce foam cell formation. If the pH was lowered to below 6 in such preparations, fusion of LDL particles was aggravated and complement-activating properties as well as foam cell induction appeared. Treatment with neuraminidase dramatically augmented these properties to yield the E-LDL described herein. It is of interest that one group of investigators has been promoting the concept that removal of sialic acid from LDL renders the molecule more atherogenic (61). Our results are quite compatible with this thought, although our in vitro data indicate that removal of sialic acid alone is not sufficient to transform LDL into an atherogenic molecule.

Finally, a most recent paper reported that in vitro lipolysis of hypertriglyceridemic serum also leads to formation of liposomes that induce foam cell formation in mouse macrophages (62). These particles may represent VLDL surface remnants, and they may represent another relevant class of atherogenic lipids.

Overall, the concept emerging from the present work satisfactorily accounts for major reported findings and explains apparent discrepancies and enigmas in the literature. It is in line with the concept that LDL, but not HDL, is atherogenic. It provides an explanation why fusion of LDL particles occurs shortly after its accumulation in the subendothelium, why complement activation occurs also at this early stage, and why monocytic infiltration then follows. It is in line with the absence of large amounts of oxidized lipids in lipoproteins isolated from plaques. It accounts for the phenomenon of foam cell formation, and shows this process to be essentially independent of oxidative processes. The novelty of the present concept does not, of course, rest on the mere finding that enzymatic modification leads to LDL aggregation and fusion; as discussed above, LDL aggregates have been shown to form under a variety of other conditions. Rather, it is stressed that of all modifications reported to date, this is the only one that endows LDL with complement-activating properties. Furthermore, LDL aggregates generated by other procedures have never clearly been shown to be taken up via the scavenger receptor pathway, and no other LDL-derived moiety has been shown to be taken up by human macrophages to an extent exceeding that observed for oxidized or acetylated LDL, as has been found for E-LDL. We would like to propose that enzymatic modification of LDL represents the missing link between subendothelial LDL deposition and initiation of atherosclerosis.

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References

1. Newman, H.A.I., and D.B. Zilversmith. 1962. Quantitative aspects of cholesterol flux in rabbit atheromatous lesions. *J. Biol. Chem.* 237:2078–2093.
2. Goldstein, J.L., and M.S. Brown. 1977. The low-density lipoprotein pathway and its relation to atherosclerosis. *Annu. Rev. Biochem.* 46:897–930.
3. Goldstein, J.L., W.R. Hazzard, H.G. Schrott, E.L. Biermann, and A.G. Motulsky. 1973. Hyperlipidemia in coronary heart disease. I. Lipid levels in 500 survivors of myocardial infarction. *J. Clin. Invest.* 53:1533–1548.
4. Schwenke, D.C., and T.E. Carew. 1989. Initiation of atherosclerotic lesions in cholesterol-fed rabbits. I. Focal increases in aterterial LDL concentration precede development of fatty streak lesions. *Arteriosclerosis.* 9:895–907.
5. Schwenke, D.C., and T.E. Carew. 1989. Initiation of atherosclerotic lesions in cholesterol-fed rabbits. II. Selective retention of LDL vs selective increase in LDL permeability in susceptible sites of arteries. *Arteriosclerosis.* 9:908–918.
6. Brown, M.S., and J.L. Goldstein. 1983. Lipoprotein metabolism in the macrophage: implications for cholesterol deposition in atherosclerosis. *Annu. Rev. Biochem.* 52:223–261.
7. Berliner, J.A., and R.G. Gerrity. 1992. Pathology of atherogenesis. *In* Molecular Genetics of Coronary Artery Disease. Candidate Genes and Processes in Atherosclerosis. A.J. Lusis,

J.I. Rotter, and R.S. Sparkes, editors. *Monogr. Hum. Genet.* 14:1–17. S. Karger AG, Basel.
8. Ross, R. 1986. The pathogenesis of atherosclerosis: an update. *N. Engl. J. Med.* 314:488–500.
9. Territo, M.C., J.A. Berliner, and M. Navab. 1992. Cellular interactions of the arterial wall. *In* Molecular Genetics of Coronary Artery Disease. Candidate Genes and Processes in Atherosclerosis. A. J. Lusis, J.I. Rotter, and R.S. Sparks, editors. *Monogr. Hum. Genet.* 14:18–34. S. Karger AG, Basel.
10. Nieminen, M.S., K. Mattila, and V. Valtonen. 1993. Infection and inflammation as risk factors for myocardial infarction. *Eur. Heart J.* 14(Suppl. K):12–16.
11. Hessler, J.R., D.W. Morel, L.J. Lewis, and G.M. Chisolm. 1983. Lipoprotein oxidation and lipoprotein-induced cytotoxicity. *Arteriosclerosis.* 3:215–222.
12. Henriksen, T., E. Mahoney, and D. Steinberg. 1981. Enhanced macrophage degradation of low density lipoprotein previously incubated with cultured endothelial cells: recognition by the receptor for acetylated low density lipoproteins. *Proc. Natl. Acad. Sci. USA.* 78:6499–6503.
13. Steinbrecher, U.P., S. Parthasarathy, D.S. Leake, J.L. Witzum, and D. Steinberg. 1984. Modification of low density lipoprotein by endothelial cells involves lipid peroxidation and degradation of low density lipoprotein phospholipids. *Proc. Natl. Acad. Sci. USA.* 81:3883–3887.
14. Zhang, H., H.J.K. Basra, and U.P. Steinbrecher. 1990. Effects of oxidatively modified LDL on cholesterol esterification in cultured macrophages. *J. Lipid Res.* 31:1361–1369.
15. Goldstein, J.L., Y.K. Ho, S.K. Basu, and M.S. Brown. 1979. Binding site on macrophages that mediates uptake and degradation of acetylated low density lipoprotein producing massive cholesterol deposition. *Proc. Natl. Acad. Sci. USA.* 76: 333–337.
16. Fogelman, A.M., I. Schechter, J. Seager, M. Hokom, J.S. Child, and P.A. Edwards. 1980. Malondialdehyde alteration of low density lipoproteins leads to cholesteryl ester accumulation in human monocyte macrophages. *Proc. Natl. Acad. Sci. USA.* 77:2214–2218.
17. Steinberg, D., S. Parthasarathy, T.E. Carew, J.C. Khoo, and J.L. Witzum. 1989. Beyond cholesterol. Modifications of low-density lipoprotein that increase its atherogenicity. *N. Engl. J. Med.* 320:915–924.
18. Haberland, M.E., and U.P. Steinbrecher. 1992. Modified low-density lipoproteins: diversity and biological relevance in atherogenesis. *In* Molecular Genetics of Coronary Artery Disease. Candidate Genes and Processes in Atherosclerosis. A.J. Lusis, J.I. Rotter, and R.S. Sparkes, editors. *Monogr. Hum. Genet.* 14:35–61. S. Karger AG, Basel.
19. Cushing, S.D., J.A. Berliner, A.J. Valente, M.C. Territo, M. Navab, F. Parhami, R. Gerrity, C.J. Schwartz, and A.M. Fogelman. 1990. Minimally modified low density lipoprotein induces monocyte chemotactic protein 1 in human endothelial cells and smooth muscle cells. *Proc. Natl. Acad. Sci. USA.* 87:5134–5138.
20. Berliner, J.A., M.C. Territo, A. Sevanian, S. Ramin, J.A. Kim, B. Bamshad, M. Esterson, and A.M. Fogelman. 1990. Minimally modified low density lipoprotein stimulates monocyte endothelial interactions. *J. Clin. Invest.* 85:1260–1266.
21. Kume, N., M.I. Cybulsky, and M.A. Gimbrone, Jr. 1992. Lysophosphatidyl-choline, a component of atherogenic lipoproteins, induces mononuclear leukocyte adhesion molecules in cultured human and rabbit arterial endothelial cells. *J. Clin. Invest.* 90:1138–1144.
22. Steinbrecher, U.P., and M. Longheed. 1992. Scavenger receptor-independent stimulation of cholesterol esterification in macrophages by low density lipoprotein extracted from human aortic intima. *Arterioscler. Thromb.* 12:608–625.
23. Kruth, H.S. 1984. Localization of unesterified cholesterol in human atherosclerotic lesions. Demonstration of filipin-positive oil red-O-negative particles. *Am. J. Pathol.* 114:201–208.
24. Chao, F.F., L.M. Amenda, E.J. Blanchette-Mackie, S.I. Scarlatos, W. Gamble, J.H. Resau, W.T. Mergner, and H.S. Kruth. 1988. Unesterified cholesterol-rich lipid particles in atherosclerotic lesions of human and rabbit aortas. *Am. J. Pathol.* 131:73–83.
25. Frank, J.S., and A.M. Fogelman. 1989. Ultrastructure of the intima in WHHL and cholesterol-fed rabbit aortas prepared by ultra-rapid freezing and freeze-etching. *J. Lipid. Res.* 30: 967–978.
26. Simionescu, N., E. Vasile, F. Lupu, G. Popescu, and N. Simionescu. 1986. Prelesional events in atherosclerosis: accumulation of extracellular cholesterol-rich liposomes in the arterial intima and cardiac valves of the hyperlipidemic rabbit. *Am. J. Pathol.* 123:109–125.
27. Kruth, H.S. 1985. Subendothelial accumulation of unesterified cholesterol. An early event in atherosclerotic lesion development. *Atherosclerosis.* 57:337–341.
28. Mora, R., F. Lupu, and N. Simionescu. 1987. Prelesional events in atherosclerosis: colocalization of apoprotein B, unesterified cholesterol and extracellular phospholipid liposomes in the aorta of hyperlipidemic rabbits. *Atherosclerosis.* 67:143–154.
29. Seifert, P.S., F. Hugo, J. Tranum-Jensen, U. Zähringer, M. Muhly, and S. Bhakdi. 1990. Isolation and characterization of a complement-activating lipid extracted from human atherosclerotic lesions. *J. Exp. Med.* 172:547–557.
30. Hoff, H.F., J. O'Neill, J.M. Pepin, and T.B. Cole. 1990. Macrophage uptake of cholesterol-containing particles derived from LDL and isolated from atherosclerotic lesions. *Eur. Heart J.* 11(Suppl. E):105–115.
31. Chatterton, J.E., M.L. Phillips, L.K. Curtiss, R.W. Milne, Y.L. Marcel, and V.N. Schumaker. 1993. Mapping apolipoprotein B on the low density lipoprotein surface by immunoelectron microscopy. *J. Biol. Chem.* 266:5955–5962.
32. Kazatchkine, M.D., and U. Nydegger. 1980. The human alternative complement pathway. *Prog. Allergy.* 30:193–222.
33. Chao, F.F., E.J. Blanchette-Mackie, V.V. Tertov, S.I. Skarlatos, Y.J. Chen, and H.S. Kruth. 1992. Hydrolysis of cholesterol ester in low density lipoprotein converts this lipoprotein to a liposome. *J. Biol. Chem.* 267:4992–4998.
34. Havel, R.J., H.A. Eder, and J.H. Bragdon. 1955. The distribution and chemical composition of ultracentrifugally separated lipoproteins in human serum. *J. Clin. Invest.* 34:1345–1353.
35. Salacinski, P.R., C. McLean, J.E. Sykes, V.V. Clement-Jones, and P.J. Lowry. 1981. Iodination of proteins, glycoproteins and peptides using a solid-phase oxidizing agent (iodogen). *Anal. Biochem.* 117:136–146.
36. Wieland, E., S. Parthasarathy, and D. Steinberg. 1993. Peroxidase-dependent metal independent oxdidation of low density lipoprotein in vitro: a model for in vivo oxidation? *Proc. Natl. Acad. Sci. USA.* 90:5929–5933.
37. Schettler, V., E. Wieland, R. Verwiebe, P. Schuff-Werner, F. Scheler, and M. Oellerich. 1994. Plasma lipids are not oxidized during hemodialysis. *Nephron.* 67:42–47.
38. Wieland, E., V. Schettler, F. Diedrich, P. Schuff-Werner,

and M. Oellerich. 1992. Determination of lipid hydroperoxides in serum: idiometry and HPLC compared. *Eur. J. Clin. Chem. Clin. Biochem.* 30:363–369.
39. El Saadani, M., H. Esterbauer, M. El-Sayed, M. Goher, A.Y. Naassar, and G. Jürgens. 1989. A spectrophotometric assay for lipid peroxidation in serum lipoproteins using a commercially available reagent. *J. Lipid. Res.* 30:627–630.
40. Denholm, E.M., and F.M. Wolber. 1991. A simple method for the purification of human peripheral blood monocytes. A substitute for sepracell MN. *J. Immunol. Meth.* 144:247–251.
41. Brown, M.S., Y.K. Ho, and J.L. Goldstein. 1980. The cholesterol ester cycle in macrophage foam cells. Continual hydrolysis and re-esterification of cytoplasmic cholesteryl esters. *J. Biol. Chem.* 255:9344–9352.
42. Greenspan, P., E.P. Mayer, and S.D. Fowler. 1985. Nile red: a selective fluorescent stain for intracellular lipid drops. *J. Cell Biol.* 100:965–973.
43. Wieland, E., A. Brandes, V.W. Armstrong, and M. Oellerich. 1993. Oxidative modification of low density lipoproteins by human polymorphonuclear leukocytes. *Eur. J. Clin. Chem. Clin. Biochem.* 31:725–731.
44. Bhakdi, S., M. Roth, and W. Nürnberger. 1984. A simple method for quantitative measurement of C3d in human plasma. *J. Immunol. Meth.* 74:79–86.
45. Hugo, F., S. Krämer, and S. Bhakdi. 1987. Sensitive ELISA for quantitating the terminal membrane C5b-9 and fluid-phase SC5b-9 complex of human complement. *J. Immunol. Meth.* 99:243–251.
46. Armstrong, V.W., A.K. Walli, and D. Seidel. 1985. Isolation, characterization, and uptake in human fibroblasts of an apo(a)-free lipoprotein obtained on reduction of lipoprotein(a). *J. Lipid Res.* 26:1314–1323.
47. Tranum-Jensen, J. 1988. Electron microscopy: assays involving negative staining. *Methods Enzymol.* 165:357–374.
48. Cathcart, M.K., D.W. Morel, and G.M. Chisolm. 1985. Monocytes and neutrophils oxidize LDL making it cytotoxic. *J. Leukoctye Biol.* 38:341–350.
49. Chao, F.F., E.J. Blanchette-Mackie, Y.J. Chen, B.F. Dickens, L.M. Berlin, L.M. Amende, S.I. Skarlatos, W. Gamble, J.H. Resau, W.T. Mergner, and H.S. Kruth. 1990. Characterization of two unique cholesterol-rich lipid particles isolated from human atherosclerotic lesions. *Am. J. Pathol.* 136: 169–179.
50. Steinberg, D. 1983. Lipoproteins and atherosclerosis. A look back and a look ahead. *Arteriosclerosis.* 3:283–301.
51. Mora, R., M. Simionescu, and N. Simionescu. 1990. Purification and partial characterization of extracellular liposomes isolated from the hyperlipidemic rabbit aorta. *J. Lipid Res.* 31: 1793–1807.
52. Vlaicu, R., F. Niculescu, H.G. Rus, and A. Cristea. 1985. Immunohistochemical localization of the terminal C5b-9 complement complex in human aortic fibrous plaque. *Atherosclerosis.* 57:163–177.
53. Seifert, P.S., F. Hugo, G.K. Hansson, and S. Bhakdi. 1989. Prelesional complement activation in experimental atherosclerosis. Terminal C5b-9 complement deposition coincides with cholesterol accumulation in the aortic intima of hypercholesterolemic rabbits. *Lab. Invest.* 60:747–754.
54. Bhakdi, S., F. Hugo, and J. Tranum-Jensen. 1990. Functions and relevance of the terminal complement sequence. *Blut.* 60:309–318.
55. Krieger, M., and J. Herz. 1994. Structures and functions of multiligand lipoprotein receptors: macrophage scavenger receptors and LDL-receptor related protein (LRP). *Annu. Rev. Biochem.* 63:601–637.
56. Acton, S., P.E. Scherer, H.F. Lodish, and M. Krieger. 1994. Expression cloning of SR-BI, a CD36-related class B scavenger receptor. *J. Biol. Chem.* 269:21003–21009.
57. Endemann, G., L.W. Stanton, K.S. Madden, C.M. Bryant, R.T. White, and A.A. Protter. 1993. CD36 is a receptor for oxidized low density lipoprotein. *J. Biol. Chem.* 268:11811–11816.
58. Sparrow, C.P., S. Parthasarathy, and D. Steinberg. 1989. A macrophage receptor that recognizes oxidized low density lipoprotein but not acetylated low density lipoprotein. *J. Biol. Chem.* 264:2599–2604.
59. Guyton, J.R., K.F. Klemp, and M.P. Mims. 1991. Altered ultrastructural morphology of self-aggregated low density lipoproteins: coalescence of lipid domains forming droplets and vesicles. *J. Lipid. Res.* 32:953–962.
60. Suits, A.G., A. Chait, M. Aviram, and J.W. Heinecke. 1989. Phagocytosis of aggregated lipoprotein by macrophages: low density lipoprotein receptor-dependent foam-cell formation. *Proc. Natl. Acad. Sci. USA.* 86:2713–2717.
61. Orekhov, A.N., V.V. Tertov, and D.N. Mukhin. 1991. Desialylated low density lipoprotein: naturally occurring modified lipoprotein with atherogeneic potency. *Atherosclerosis.* 86: 153–161.
62. Chung, B.H., G. Tallis, V. Yalamoori, G.M. Anantharamaiah, and J.P. Segrest. 1994. Liposome-like particles isolated from human atherosclerotic plaques are structurally and compositionally similar to surface remnants of triglyceride-rich lipoproteins. *Arterioscler. Thromb.* 14:622–635.