Modification of low density lipoproteins by polymorphonuclear cell elastase leads to enhanced uptake by human monocyte-derived macrophages via the low density lipoprotein receptor pathway

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Abstract In previous studies we reported that polymorphonuclear cell (PMN) elastase cleaves apoB-100 of human plasma low density lipoprotein (LDL) into seven or eight large M_r fragments (1, Polacek, D., R.E. Byrne, G.M. Fless, and A.M. Scanu. 1986. *J. Biol. Chem.* **261**: 2057–2063). In the present studies we examined the interaction of native and elastase-digested LDL (ED-LDL) with primary cultures of human monocyte-derived macrophages (HMD-M). For this purpose LDL was digested with purified PMN elastase, re-isolated by ultracentrifugation at d 1.063 g/ml to remove the enzyme, and radiolabeled with ^{125}I. At all LDL concentrations in the medium, the degradation of ^{125}I-labeled ED-LDL was 1.5- to 2.5-fold greater than that of ^{125}I-labeled native LDL, and for both lipoproteins species it was further enhanced by prior incubation of the cells in autologous lipoprotein-deficient serum (ALPDS). ED-LDL incubated with HMD-M in a medium containing [^{14}C]oleate stimulated cholesteryl [^{14}C]oleate formation 2- to 3-fold more than native LDL. In competitive degradation experiments, unlabeled ED-LDL did not inhibit the degradation of ^{125}I-labeled acetylated LDL, whereas it caused a 90% inhibition of the degradation of ^{125}I-labeled native LDL. At 4°C, the binding of both ^{125}I-labeled native and ^{125}I-labeled ED-LDL was specific and of a high affinity. At saturation (B_{max}), the binding of ^{125}I-labeled ED-LDL was 2-fold higher (68 ng/mg cell protein) than that of ^{125}I-labeled native LDL (31 ng/mg), with K_d values of 6.5×10^{-8} M and 2.1×10^{-8} M, respectively. A possible explanation of the binding data was provided by electrophoretic analyses suggesting that ED-LDL was twice the size of native LDL and thus potentially capable of delivering proportionately more cholesterol to the cells. Taken together, the results indicate that *1*) digestion of LDL by purified PMN elastase results in a greater mass of ED-LDL (relative to native LDL) being degraded per unit time by HMD-M; *2*) uptake of ED-LDL occurs via the LDL receptor; and *3*) LDL digested by PMN elastase undergoes a physical change that may be responsible for its unique interactions with HMD-M. We speculate that if this process were to occur in vivo during an inflammatory process, macrophages could acquire excess cholesterol and be transformed into foam cells which are considered to be precursors of the atherosclerotic process.—**Polacek, D., R.E. Byrne, and A.M. Scanu.** Modification of low density lipoproteins by polymorphonuclear cell elastase leads to enhanced uptake by human monocyte-derived macrophages via the low density lipoprotein receptor pathway. *J. Lipid Res.* 1988. **29:** 797–808.

Supplementary key words PMN elastase and LDL • proteolytic enzymes • monocyte-derived macrophage receptors • LDL receptor

In a previous report we showed that when human low density lipoprotein (LDL) is incubated with either activated human polymorphonuclear cells (PMN) or purified PMN elastase, it undergoes proteolysis as indicated by the degradation of apoB-100 into seven or eight large M_r components. There are several reports in the literature indicating that LDL modified by either chemical agents or oxidation is no longer taken up by the physiological LDL receptor pathway. On the other hand, LDL digested by proteolytic enzymes such as trypsin (2,3), plasmin (4), and kallikrein (5) retain affinity for the LDL receptor. Based on these observations we considered it of interest to determine the biological behavior of PMN elastase-modified LDL (ED-LDL), using as a model system primary cultures of human monocyte-derived macrophages (HMD-M). We selected these cells because we wanted to work in a homologous lipoprotein-cell culture system and use primary cells having both the LDL and scavenger receptors. It is known that mouse peritoneal macrophages (MPM) have few LDL receptors and that these receptors have a very low affinity

Abbreviations: LDL, human low density lipoprotein; HMD-M, human monocyte-derived macrophages; DMEM, Dulbecco's modified Eagle's medium; ALPDS, autologous lipoprotein-deficient human serum; HEPES, 4-(2-hydroxyethyl)-1-piperazine-ethanesulfonic acid; PBS, phosphate-buffered saline; PBS(CMF), phosphate-buffered saline minus calcium and magnesium; PBS(BSA), PBS(CMF) containing 0.2% bovine serum albumin; PMN, human polymorphonuclear cells; ED-LDL, human low density lipoprotein that has been digested by purified human polymorphonuclear cell elastase; ED_{15}-LDL, ED_{60}-LDL, ED_{120}-LDL, and ED_{240}-LDL are abbreviations for human low density lipoprotein that has been digested by purified human polymorphonuclear cell elastase for 15, 60, 120, and 240 min, respectively; DFP, diisopropyl fluorophosphate; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; GGE, gradient gel electrophoresis.

for human LDL (6,7). Moreover, other human established macrophage cell lines, such as the U-937 and HL-60 lines described by Via et al. (8), have no scavenger receptor activity, and Tabas, Weiland, and Tall (9) have reported that the J774 mouse macrophage line is anomalous in that these cells accumulate cholesteryl esters following incubation with native, unmodified LDL.

Since the emphasis of our current work was on the effect of proteolysis, we wanted to separate it from effects due to oxidation. According to Raymond, Reynolds, and Swanson (10), LDL can undergo oxidation after exposure to stimulated leukocytes during inflammation, and Cathcart, Morel, and Chisolm (11) have shown that the oxidation of LDL can occur after exposure to unstimulated PMN. It has also been observed that oxidized LDL is degraded more readily by macrophages and can induce cholesteryl ester accumulation in these cells. To overcome these complications in the current study, we made use of LDL incubated with purified PMN elastase, which we demonstrated previously to be free of oxidized species. In addition, we have cultured HMD-M in DMEM, a medium that does not promote the oxidation of LDL (containing no copper and little iron) (12).

The purpose of this report is to show that LDL digested by PMN elastase when incubated with HMD-M behaves in a manner distinct from native LDL and that this difference is likely attributable to the proteolytic cleavage of apoB-100.

MATERIALS AND METHODS

Materials

Sodium [^{125}I]iodide and [1-^{14}C]oleic acid were from Amersham Corp; Dulbecco's modified Eagle's medium (DMEM) containing 25 mM HEPES, L-glutamine, and 4500 mg/l glucose, and phosphate-buffered saline minus calcium and magnesium [PBS(CMF)] were from Gibco Laboratories; Plasmagel was from Cellular Products, Inc., Buffalo, NY; DFP was from Aldrich; bovine serum albumin Fraction V, soybean trypsin inhibitor, human serum albumin, and kallikrein were products of Sigma; Ficoll-Paque was from Pharmacia; methoxysuccinyl-Ala-Ala-Pro-Val-4-methylcoumaryl-7-amide was from Vega Biochemical.

Preparation of LDL from human plasma

The blood of healthy human donors was collected into a solution containing EDTA (1.5 g/l) and soybean trypsin inhibitor (0.04 g/l), and the plasma was separated by centrifugation first at 4,080 *g* for 30 min at 4°C, followed by another centrifugation at 12,000 *g* for 10 min at 4°C. The LDL (d 1.025–1.045 g/ml) was isolated by sequential ultracentrifugal flotation. After dialysis against 1,000 volumes of 0.15 M NaCl, 0.01% EDTA, pH 7.4, the LDL was passed through a 0.45 μm sterilizing filter and stored under nitrogen in the dark at 4°C until use.

Protein concentrations were determined by the method of Lowry et al. (13) using bovine serum albumin as standard.

Chemical modifications (acetylation and succinylation) of LDL

LDL in 0.15 M NaCl was acetylated using the general procedure of Chu, Crary, and Bergdoll (14) by addition of a 60-fold molar excess of acetic anhydride to apoB lysine (43 μmol of reagent/mg of protein). The reaction was carried out in an ice bath with constant stirring and the pH was maintained at 8.0 by the addition of 0.1 N NaOH. The reagent was added in two separate aliquots in order to control the reaction rate. The reaction was judged complete when all of the reagent was added and the pH remained essentially constant. After acetylation, the preparation was extensively dialyzed against 0.15 M NaCl, 0.01% EDTA, passed through a 0.45 μm sterilizing filter, and stored under nitrogen in the dark at 4°C until use. Acetylation was confirmed by migration on agarose gel electrophoresis (15).

Succinylation of LDL was performed in exactly the same way as the acetylation except that succinic anhydride was used.

Preparation of PMN elastase

Elastase was purified from human PMN (1) and assayed with the substrate methoxysuccinyl-Ala-Ala-Pro-Val-4-methylcoumaryl-7-amide as described previously by Polacek et al. (1). The standard assay mixture consisted of 90 nmol of substrate in 0.025 M Tris–HCl, 0.5 M NaCl, 0.02% NaN_3, pH 7.4, in a final volume of 1.5 ml. One unit of elastase activity was defined as that quantity of enzyme that cleaved 1 nmol of substrate/min at 37°C under standard assay conditions.

Preparation of PMN elastase-digested LDL (ED-LDL)

LDL (20 mg of protein) was incubated under sterile conditions and under nitrogen with 13 units of PMN elastase in a total volume of 3.0 ml of 0.15 M NaCl, 0.01% EDTA, 0.025 M Tris–HCl, pH 8.0, in a shaking water bath. At timed intervals (15–240 min) the reaction was stopped by removing samples and inactivating the enzyme by the addition of DFP (1.0 mM final concentration). Standard ED-LDL was incubated for 120 min.

ED-LDL was concentrated by ultracentrifugal flotation at d 1.063 g/ml at 224,000 *g*, 5°C, for 20 hr. This procedure also served to separate the enzyme from the lipoprotein by sedimentation of the former to the bottom of the tube (1). ED-LDL was then extensively dialyzed against 0.15 M NaCl, 0.01% EDTA, pH 7.4, passed through a 0.45 μm sterilizing filter and stored under nitrogen in the dark at 4°C until use. Extent of proteolysis was assessed on SDS-GGE.

Isolation and culture of HMD-M

Freshly drawn human blood (180 ml) was collected at a final heparin concentration of 15 units/ml. Aliquots of blood (30 ml each) were mixed with 15 ml of Plasmagel in 60-ml sterile, plastic syringes and left to stand on their plungers. The red blood cells were allowed to settle, after which time (30 min, 25°C) the upper leukocyte-rich layer was expelled through sterile tubing by slowly pushing up on the plungers. This fraction was then centrifuged at 400 *g* for 10 min at 4°C in order to pellet the leukocytes. Pellets were resuspended in PBS (CMF), 0.1% BSA, 15 mM glucose and immediately loaded into a Beckman J-21 Elutriation System. Monocyte fractions were eluted essentially according to method BB of Fogelman et al. (16). The monocyte-containing fractions were pooled, centrifuged at 150 *g* for 10 min at 4°C, and the pellets were resuspended in PBS (CMF), 0.1% BSA to a final volume of 50 ml. Two 25-ml fractions were then underlaid with 15 ml of Ficoll-Paque and centrifuged at 400 *g*, 18°C, for 40 min. The mononuclear cells at the interface were removed and resuspended in six volumes of cold PBS(CMF), 0.1% BSA and centrifuged again at 400 *g*, 4°C, for 10 min. The cell pellets were resuspended in 6 ml of DMEM, counted, and finally resuspended in DMEM (20% autologous serum) to a final concentration of 0.6×10^6 cells/ml; 1.0 ml was plated into each well of Linbro 12-well tissue culture plates (area per well = 4.5 cm^2). The cells were placed in a 37°C humidified-air incubator (95% air, 5% CO_2) and allowed to attach for 30 min, after which time the wells were rinsed twice with serum-free DMEM and finally overlaid with 1.0 ml of DMEM (20% autologous serum). Cultures were refed 24 hr later and thereafter, every 3 days.

Preparation of human autologous serum and lipoprotein-deficient serum (ALPDS)

Human autologous serum was prepared from 125 ml of whole blood from each monocyte donor. Blood was collected in 30-ml Corex tubes and allowed to clot at room temperature for 30 min before the tubes were placed at 4°C for 1 hr. The tubes were then centrifuged at 4,000 rpm, 4°C, for 15 min in a Sorvall SS-34 rotor. The serum layer was removed and recentrifuged for an additional 10 min. Finally, the serum was passed through a 0.45 μm sterilizing filter and stored at 4°C until use.

For the preparation of ALPDS, the procedure outlined above was followed, but then the serum was brought to d 1.21 g/ml with NaBr and centrifuged at 224,000 *g*, 5°C for 48 hr, after which time the top half of the centrifuge tube was sliced off and the bottom fractions were pooled. This ALPDS was then extensively dialyzed against 0.15 M NaCl, passed through a 0.45 μm sterilizing filter and stored at 4°C until use.

Cholesterol esterification assays

Monolayers of macrophages were washed twice in serum-free DMEM and then incubated for 16 hr at 37°C with 0.2 mM [^{14}C]oleate/albumin in DMEM in the absence and presence of various lipoproteins (17). After incubation, the monolayers were washed, the cells were harvested by scraping, and the cellular cholesteryl esters were isolated by thin-layer chromatography and quantitated by liquid scintillation counting with [^{3}H]cholesteryl oleate as internal standard. Final results were expressed as nmoles of [^{14}C]oleate incorporated into cholesteryl ester/mg of cell protein. Base level incorporation, measured in the absence of lipoprotein, was not subtracted from values obtained in the presence of lipoprotein.

Binding of ^{125}I-labeled LDL to HMD-M at 4°C

Lipoprotein binding assays at 4°C were performed on confluent monolayers of macrophages without preincubation in ALPDS. Assays were carried out essentially according to Innerarity, Pitas, and Mahley (18). Briefly, macrophage monolayers were washed with DMEM (10% ALPDS) and cooled to 4°C. The medium was then removed and pre-cooled ^{125}I-labeled LDL in DMEM (10% ALPDS) was added with and without unlabeled competing LDL. Disheswere incubated at 4°C, with rocking, for 3 hr. The cells were then rapidly washed three times with 1.0 ml of cold PBS(BSA), followed by two 10-min incubations with the same buffer, and finally a rapid wash using cold PBS(CMF). The cell monolayer was then dissolved in 0.5 ml of 0.1 N NaOH. Aliquots were taken for determination of ^{125}I cpm by gamma counting and protein by Lowry protein determination (13). Radioactivity was normalized per mg of cell protein. Experimentally obtained points for total and nonspecific binding (binding in the presence of a 30-fold excess of unlabeled lipoprotein) were plotted and theoretical curves were drawn for those points. Values for the equilibrium dissociation constant and the maximal binding at saturation were derived from a curve that was the result of subtracting the curve for nonspecific binding from that for total binding.

Electrophoretic procedures

For the separation of apoB-100 and apoB fragments, SDS (3–6%) GGE analyses were performed using a modified Laemmli discontinuous buffer system (19). Routinely, the separating gel, in 0.45 M Tris–HCl, pH 8.8, consisted of a stepwise 3–6% polyacrylamide gradient in 0.2% SDS superimposed on a 0–20% sucrose gradient. Polymerization of the gel was accomplished by addition of ammonium persulfate and N,N,N',N-tetramethylethylenediamine to final concentrations of 0.05% (w/v) and 0.0003% (v/v), respectively, in each solution. The stacking gel, in 0.067 M Tris–HCl, pH 6.8, contained 2.75% polyacrylamide. The

running buffer was 0.05 M Tris, 0.38 M glycine–HCl, 0.2% SDS, pH 8.3. Gels were stained in 0.25% Coomassie blue R 250 in 25% isopropanol (10% acetic acid) and destained for 30 min in 25% isopropanol (10% acetic acid), followed by final destaining in 10% acetic acid until the background was clear.

Nondenaturing polyacrylamide gradient gel electrophoresis of LDL was performed using 2.5–10% linear gradient gels in 0.09 M Tris, 0.08 M borate, 0.003 M EDTA, pH 8.3. Following electrophoresis for 2700 V-hr, gels were stained and destained as described above.

Agarose gel electrophoretic analyses of lipoproteins were performed according to Noble (15) using precast Agarose Universal Electrophoresis Film (Corning, Palo Alto, CA) in a Corning cassette electrophoresis cell filled with 0.05 M barbital buffer containing 0.035% EDTA, pH 8.6, and run according to the manufacturer's instructions. The films were stained with fat red 7B for lipids.

Cross-linking of LDL with dimethylsuberimidate

LDL was dialyzed against 0.2 M triethanolamine HCl, pH 8.5, and reacted with dimethylsuberimidate (3:1, w/w, protein:cross-linker) for 2 hr at 37°C as described by Edelstein, Halari, and Scanu (20) for the cross-linking of high density lipoprotein. After the reaction, the excess dimethylsuberimidate was removed by dialysis. At this ratio of protein:cross-linker, all of the apoB-100 was converted to higher M_r forms as assessed by SDS-GGE.

Iodination of LDL

Iodination of LDLs was performed as described in a previous publication (1). Final specific activities ranged from 200 to 350 cpm/ng of LDL protein. In the case of ED-LDL and acetylated LDL, the iodination was performed on the samples after the proteolytic and chemical modifications, respectively.

Degradation of ^{125}I-labeled LDL by HMD-M

Degradation experiments were performed essentially according to the procedure of Goldstein, Basu, and Brown (21). The incubation medium consisted of DMEM containing human serum albumin (20 mg/ml) rather than LPDS and, unless specified, cultures of HMD-M were not preincubated in ALPDS prior to the experiments.

Classification and viability of cells

HMD-M classification and viability were determined as described by Fogelman et al. (22). Due to the frequent medium changes and the washes prior to the experiments, the cells were > 99% monocyte macrophages before the test lipoproteins were added. There was no change in the viability of the cells after the experiments.

Compositional analyses of LDL

Compositional analyses of native and ED-LDL were performed according to the methods of Lowry et al. (13) for protein, Bartlett (23) for phospholipid, Gallo et al. (24) for free and esterified cholesterol, and Wahlefeld (25) for triglycerides.

RESULTS

Properties of ED-LDL

The migration of ED-LDL on agarose gel electrophoresis was indistinguishable from that of native LDL, whereas that of acetylated LDL, a more negatively charged particle, was faster **(Fig. 1).**

Rate zonal flotation of native and ED-LDL in a linear 7.5–30% (d 1.06–1.29 g/ml) NaBr gradient showed identical profiles **(Fig. 2)**, suggesting that no significant changes in the size or density of ED-LDL as a result of the proteolysis had occurred.

In accord with the physical studies above, the chemical compositions of native and ED-LDL were essentially the same (see **Table 1**). For example, the cholesterol/protein ratio of native LDL was 2.33 compared to a ratio of 2.35 for ED-LDL.

Degradation of ^{125}I-labeled native and ED-LDL by HMD-M

HMD-M were grown in DMEM (20% autologous serum) for 8 days. On day 8, cells were washed and incubated for 5 hr, 37°C, in DMEM, human serum albumin (20 mg/ml), and the indicated concentrations of ^{125}I-labeled native or ED-LDL (see Methods for details). The degra-

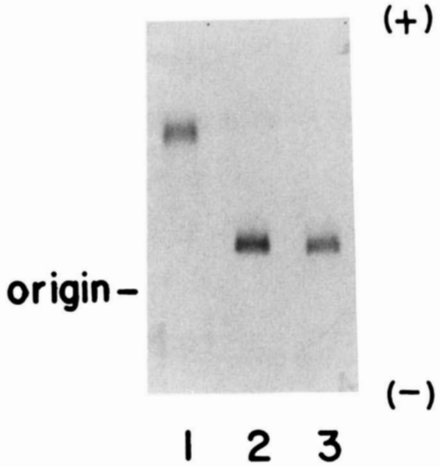

Fig. 1. Agarose gel electrophoretic patterns of native LDL, acetylated LDL, and ED-LDL. Each LDL sample (2 μg) was applied to a prepared agarose film (1%) and electrophoresed in 0.065 M barbital, 0.035% EDTA, pH 8.6. Lipoproteins were fixed and stained with fat red 7B. Lane 1, acetylated LDL; lane 2, native LDL; lane 3, ED-LDL.

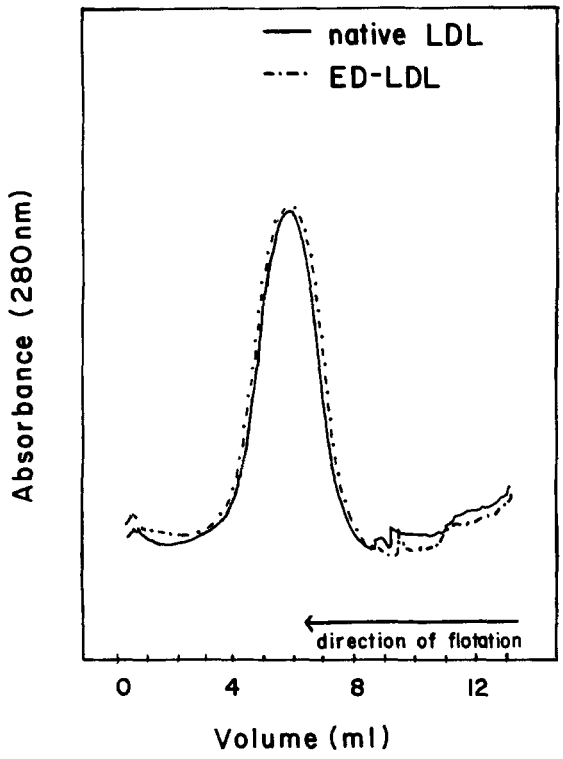

Fig. 2. Rate zonal flotation of native LDL and ED-LDL. Two mg (0.5 ml) of an LDL solution was made 40% NaBr, layered under a linear 7.5–30% NaBr gradient (total vol = 13.4 ml), and centrifuged for 3.25 hr at 35,000 rpm in a SW40 rotor (Beckman) at 20°C. The gradient was fractionated and OD_{280} was monitored by pumping out at 1.0 ml/min.

dation curve (**Fig. 3**) for ^{125}I-labeled native LDL was biphasic, one phase representing a saturable high-affinity process between 0 and 20 μg of LDL protein/ml, and a low-affinity phase, saturable at LDL concentrations greater than 20 μg of protein/ml. The degradation curve for ^{125}I-labeled ED-LDL also exhibited two components: one of high affinity from 0 to 20 μg of protein/ml, and the other, apparently nonsaturable and linearly increasing up to 300 μg of protein/ml (higher values not shown).

At the same LDL concentrations, the degradation of ED-LDL was always greater than that of native LDL. The rate of degradation of ED-LDL was 1.5- to 2.0-fold that of native LDL throughout the high-affinity portion of the degradation curve. At LDL concentrations > 50 μg of protein/ml, the rate of degradation of ED-LDL increased linearly while that of native LDL remained constant. At 200 μg of protein/ml, the amount of ED-LDL degraded over 5 hr was approximately twice that of native LDL.

When HMD-M were preincubated for 48 hr in DMEM (10% ALPDS), the rates of degradation for both native and ED-LDL were doubled, indicating that the uptake mechanism for ED-LDL was subject to regulation and to the same extent as the macrophage LDL receptor for native LDL.

Stimulation of cholesteryl ester formation in HMD-M incubated with native and ED-LDL

To evaluate the capacity of ED-LDL to stimulate cholesteryl ester formation in HMD-M, cells were incubated with either native or ED-LDL in the presence of [^{14}C]oleate/albumin complexes and the incorporation of [^{14}C]oleate into cellular cholesteryl esters was measured. As shown in **Fig. 4**, ED-LDL stimulated the formation of cholesteryl [^{14}C]oleate approximately two- to three-fold over that observed when cells were incubated with native LDL. Macrophages incubated with acetylated LDL at 100 μg of protein/ml showed a 12-fold stimulation of cholesteryl ester formation (data not shown). The dose–response curve for ED-LDL exhibited a high-affinity component from 0 to 20 μg of LDL protein/ml and a lower affinity, linear component from 20 to at least 400 μg of protein/ml (the highest LDL concentration used in these studies).

Yamamoto, Ranganathan, and Kottke (5) digested LDL with kallikrein and then assayed the capacity of this modified LDL to stimulate cholesteryl ester formation in mouse peritoneal macrophages (MPM). These authors found that kallikrein digestion of LDL had no effect on cholesterol esterification in their cell system. Kallikrein digestion of LDL generates a particle in which apoB-100 is digested into four to six lower M_r fragments ranging from approximately 130,000 to 400,000. We compared kallikrein-digested and ED-LDL with regard to their effect on cholesterol esterification in HMD-M. In agreement with the findings of Yamamoto et al. (5) with MPM, we found that kallikrein digestion of LDL had no effect on its capacity to stimulate cholesterol esterification in HMD-M (data not shown). In turn, ED-LDL caused the expected two- to three-fold stimulation of cholesterol esterification.

TABLE 1. Chemical composition of native LDL and ED-LDL

	Protein	Phospholipid	Esterified Cholesterol	Free Cholesterol	Triglyceride
			% by weight		
Native LDL	21.4 ± 0.3[a]	23.4 ± 0.5	39.8 ± 0.6	10.1 ± 0.1	5.3 ± 0.2
ED-LDL	20.6 ± 0.3	24.6 ± 0.7	38.6 ± 0.4	9.8 ± 0.1	6.4 ± 0.4

[a]Values are the average of duplicate determinations ± the average deviation.

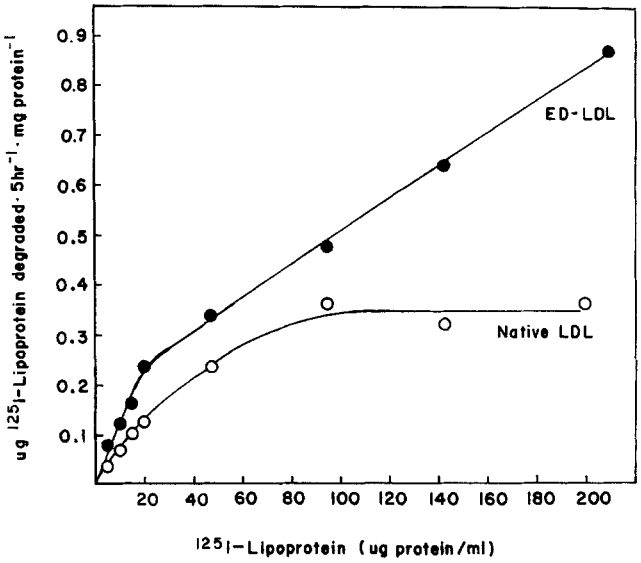

Fig. 3. Degradation of ^{125}I-labeled native LDL (○) and ^{125}I-labeled ED-LDL (●) by HMD-M. Normal monocytes were cultured in DMEM (20% autologous serum) for 8 days. The medium was removed, the cells were washed twice in serum-free DMEM, and then overlaid with 1.0 ml of DMEM, human serum albumin (20 mg/ml), and either ^{125}I-labeled native LDL or ^{125}I-labeled ED-LDL. After incubation for 5 hr at 37°C, the amount of ^{125}I-labeled, TCA-soluble, non-iodide material in the medium was determined. Values for spontaneous degradation of ^{125}I-labeled lipoproteins (in the absence of cells) were subtracted. Values are the average of duplicate incubations.

Relationship between extent of LDL digestion by elastase and LDL degradation by HMD-M

LDL was digested under incubation conditions described in Methods up to a final time point of 240 min. Samples of digested LDL were removed at 15, 60, 120, and 240 min, after which time DFP (1 mM final concentration) was added, and the samples were re-isolated by centrifugation (see Methods). The samples were radioiodinated and their degradation was assessed after a 5-hr incubation at 37°C with 14-day-old HMD-M (see Methods for experimental details). Progressive digestion of LDL with elastase (see **Fig. 5**) resulted in the disappearance of apoB-100 and the accumulation of lower M_r bands. Shown in **Fig. 6** are the degradation curves for ^{125}I-labeled native LDL, acetylated LDL, ED_{15}-LDL, ED_{60}-LDL, and ED_{240}-LDL. The degradation of ^{125}I-labeled native LDL reached a plateau at an extracellular lipoprotein concentration of approximately 50 μg of protein/ml, and that of ^{125}I-labeled acetylated LDL, at 20 μg of protein/ml. All of the ^{125}I-labeled ED-LDLs exhibited an overall similar pattern of degradation consisting of a high-affinity component at LDL concentrations between 1 and 20 μg of protein/ml, followed by a lower affinity, nonsaturable component up to at least 200 μg of protein/ml. Of the elastase-digested samples, only ED_{15}-LDL showed an SDS-GGE pattern containing a significant apoB-100 band (see Fig. 5); this sample was also degraded by HMD-M at a significantly slower rate than the LDLs digested with elastase for longer periods of time. On SDS-GGE, ED_{60}-LDL had a faint apoB-100 band, whereas ED_{120}-LDL and ED_{240}-LDL contained no band corresponding to apoB-100; these samples were all degraded by HMD-M at nearly the same rate. The major difference among the gel patterns of ED_{60}-LDL, ED_{120}-LDL, and ED_{240}-LDL was the decrease in larger M_r (> 200,000) fragments concomitant with the increase in lower M_r (approximately 60,000 to 200,000) fragments. Taken together, the results suggest that: *1*) digestion of apoB-100 by elastase results in enhanced degradation of LDL by HMD-M, and *2*) the enhanced degradation of LDL by HMD-M is not affected further by increasing the length of incubation of LDL with elastase from 60 up to 2540 min, despite changes in the apoB patterns during this digestion period.

Identification of receptor responsible for degradation of ED-LDL by HMD-M

Competitive degradation studies. In order to determine whether the native LDL receptor or the scavenger receptor was involved in the uptake and degradation of ED-LDL by HMD-M, we performed a series of competitive degradation experiments using cells from the same donor. In one experiment, ^{125}I-labeled native LDL (20 μg of protein/ml) was incubated for 5 hr at 37°C with HMD-M in the presence of increasing concentrations of unlabeled native, acetylated, or ED-LDL (**Fig. 7a**). After 5 hr the amount of TCA-soluble, non-iodide counts in the medium was de-

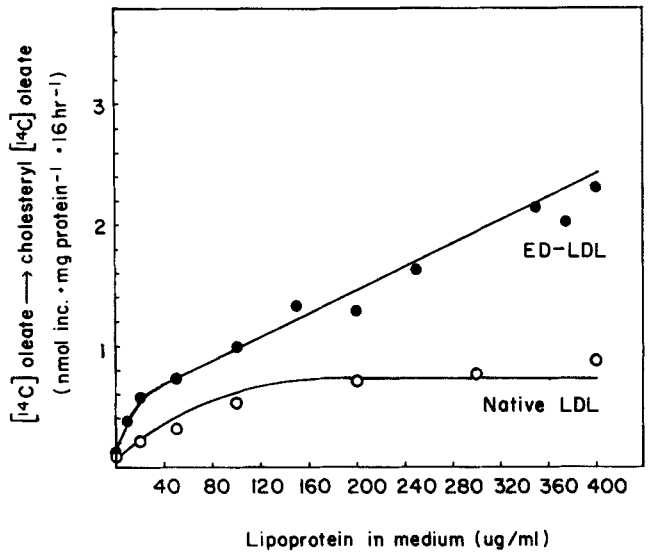

Fig. 4. Stimulation of cholesteryl ester formation in HMD-M incubated with native or ED-LDL. Monocytes were cultured for 8 days in DMEM (20% autologous serum). On day 8, cells were washed twice in serum-free DMEM and received 1.0 ml DMEM containing 0.1 mM [^{14}C]oleate/albumin (8500 dpm/nmol of oleate) and the indicated concentrations of either native LDL or ED-LDL. After incubation for 16 hr at 37°C, cells were washed and harvested and the cellular content of cholesteryl [^{14}C]oleate was determined as described in Methods. Each data point represents the average of duplicate incubations.

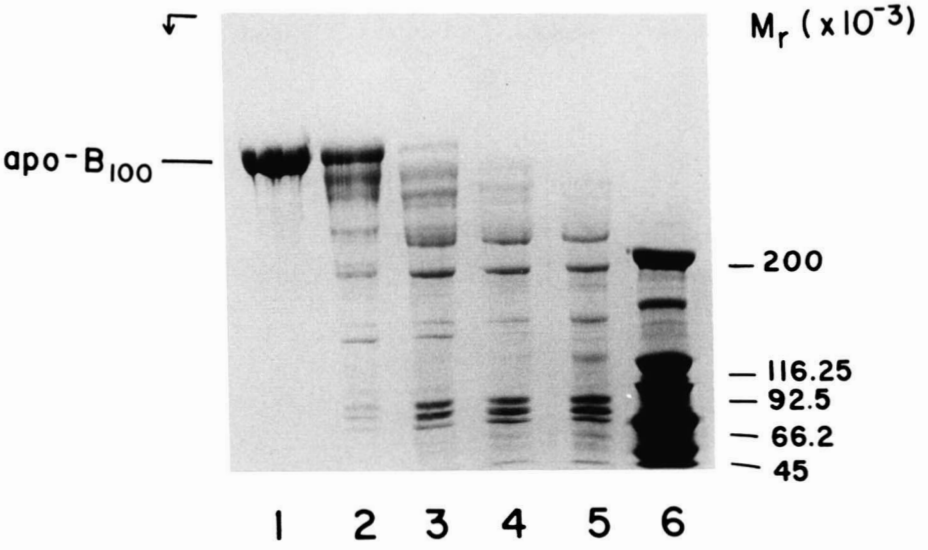

Fig. 5 Time-dependent proteolysis of apoB-100 of LDL by human PMN elastase. LDL (7 mg/ml) in 0.15 M NaCl, 0.01% EDTA, pH 7.4, was incubated with elastase (0.65 units/mg of LDL protein) in the presence of 25 mM tris, pH 8.0, for the indicated times (for details, see Methods). At each time point an aliquot was removed and DFP (1.0 mM final concentration) was added to stop the reaction. The digested LDL was then brought to d 1.063 g/ml with NaCl and centrifuged at 224,000 *g*, 5°C, for 24 hr. The refloated LDL was dialyzed against 0.15 M NaCl, 0.01% EDTA, pH 7.4, filter-sterilized, and an aliquot (25 μg of protein) was run on GGE (3–6%) in the presence of 0.2% SDS using a modified Laemmli discontinuous buffer system (19). Lane 1, native LDL; lane 2, ED_{15}-LDL; lane 3, ED_{60}-LDL; lane 4, ED_{120}-LDL; lane 5, ED_{240}-LDL; lane 6, Bio-Rad high M_r standards: myosin (200,000); beta galactosidase (116,250); phosphorylase b (97,000); bovine serum albumin (66,200); and ovalbumin (42,500).

termined (see Methods). Incubation in the presence of increasing amounts of native LDL (Fig. 7a, open circles) or ED-LDL (closed circles) resulted in a dose-dependent inhibition of ^{125}I-labeled native LDL uptake and degradation. Interestingly, the concentration of unlabeled ED-LDL required to produce half-maximal inhibition was approximately two-fold less than that of unlabeled native LDL.

At a 20-fold protein excess (the maximum used in this study), acetylated LDL (open triangles) inhibited the degradation of ^{125}I-labeled native LDL by only 20%.

Neither native nor ED-LDL competed with the high-affinity uptake and degradation of ^{125}I-labeled acetylated LDL via the scavenger receptor on HMD-M. In contrast, unlabeled acetylated LDL was an effective competitor (data not shown).

In the experiment shown in Fig. 7b, ^{125}I-labeled ED-LDL was incubated in the presence of increasing concentrations of unlabeled native, ED-LDL, or acetylated LDL. Native LDL (closed circles) or ED-LDL (open circles) inhibited the degradation of ^{125}I-labeled ED-LDL in a dose-dependent fashion.

Moreover, unlabeled acetylated LDL (open triangles) at a 20-fold protein excess inhibited the degradation of ^{125}I-labeled ED-LDL by only 25%.

Taken together, these studies strongly suggest that ED-LDL enters HMD-M via the LDL receptor and not the

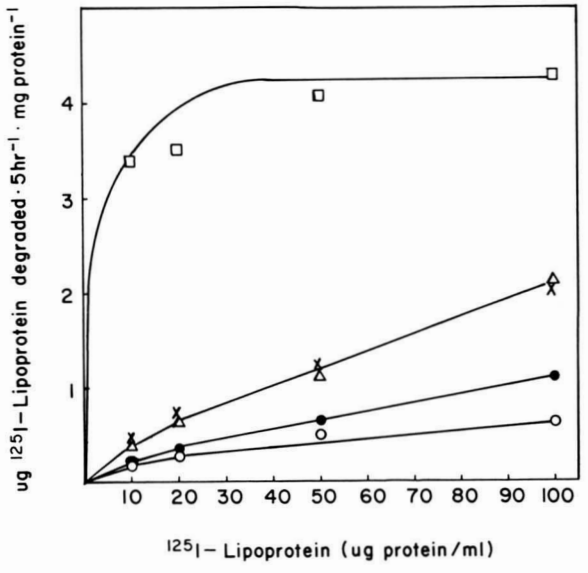

Fig. 6. Degradation of ^{125}I-labeled native LDL (O), ^{125}I-labeled ED_{15}-LDL (●), ^{125}I-labeled ED_{60}-LDL (△), ^{125}I-labeled ED_{240}-LDL (X), and ^{125}I-labeled acetylated LDL (□) by HMD-M. Monocytes were cultured in DMEM (20% autologous serum) for 14 days. The medium was removed, the cells were washed twice in serum-free DMEM and then overlaid with 1.0 ml of DMEM, human serum albumin (20 mg/ml), and the indicated concentrations of the above ^{125}I-labeled LDLs. After incubation for 5 hr at 37°C, the amount of ^{125}I-labeled, TCA-soluble, non-iodide material in the medium was determined. Values are the average of duplicate incubations.

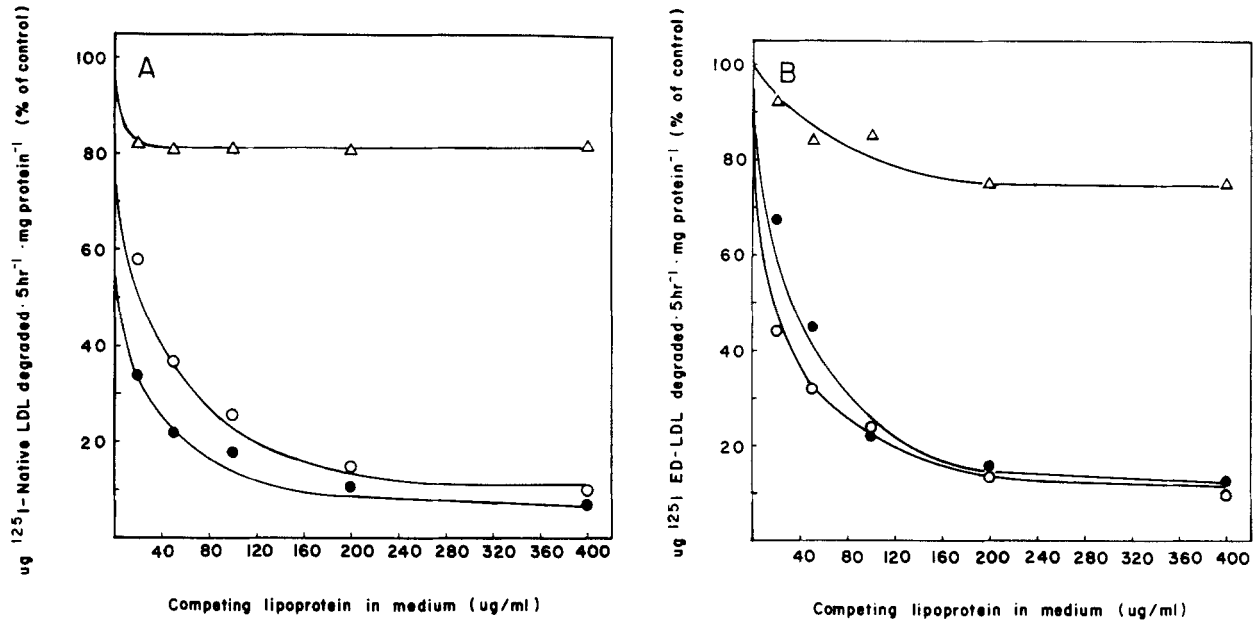

Fig. 7. Panel A: Competition by unlabeled native LDL (○), ED-LDL (●), and acetylated LDL (△), for the degradation of ^{125}I-labeled native LDL. Monocytes were cultured for 11 days in DMEM (20% autologous serum). The medium was removed, the cells were washed twice in serum-free DMEM and 1.0 ml of DMEM, human serum albumin (20 mg/ml), 20 μg of ^{125}I-labeled native LDL and the indicated concentration of unlabeled lipoprotein was added. After incubation for 5 hr at 37°C, the amount of ^{125}I-labeled, TCA-soluble, non-iodide material in the medium was determined. The 100% value for the degradation of ^{125}I-labeled native LDL in the absence of competing lipoprotein was 158 ng/mg of cell protein per 5 hr. Values are the average of duplicate incubations. Panel B: Competition by native LDL (●), ED-LDL (○), and acetylated LDL (△) for the degradation of ^{125}I-labeled ED-LDL. The 100% value for the degradation of ^{125}I-labeled ED-LDL in the absence of competing lipoprotein was 242 ng/mg cell protein per 5 hr.

scavenger receptor. In addition, unlabeled ED-LDL at one- to five-fold protein excesses resulted in more effective inhibition of ^{125}I-labeled native LDL degradation than did unlabeled native LDL, eg., in Fig. 7a, 120 μg/ml of unlabeled native LDL was required to inhibit the degradation of ^{125}I-labeled native LDL by 80%, whereas only 60 μg/ml of unlabeled ED-LDL produced the same degree of inhibition.

Equilibrium binding studies at 4°C. To characterize further the interaction between ED-LDL and HMD-M, we carried out 4°C binding experiments using native and ED-LDL labeled with ^{125}I. The cell surface binding of ^{125}I-labeled native LDL to HMD-M was a saturable, specific, and high affinity process (**Fig. 8a**). In order to measure quantitatively the affinity of the LDLs for the macrophage receptors and to determine the maximum amount of ^{125}I-labeled LDL bound/mg of cell protein, we plotted the total amount of ^{125}I-labeled LDL bound against the concentration of ^{125}I-labeled LDL in the medium. Nonspecific binding was determined as the amount of ^{125}I-labeled LDL bound in the presence of a 30-fold protein excess of unlabeled LDL. The data were then fitted to the equation of a simple thermodynamic equilibrium for the total binding and a linear equation for the nonspecific binding. Then, the specific binding was obtained by subtracting the nonspecific binding curve from the total binding curve. The maximal binding (B_{max}) and dissociation constant (K_d), were calculated from the specific binding curve. As shown in Fig. 8a and 8b, the agreement between the experimental points and the theoretical curves calculated from these parameters is excellent, thereby showing that the binding is indeed consistent with a rapid, reversible thermodynamic equilibrium binding. For native LDL, B_{max} was approximately 31 ng of LDL protein/mg of cell protein, and the K_d was approximately 2.1×10^{-8} M (M_r 514,000 for apoB-100). Thus our data show that a simple class of high-affinity binding sites is responsible for the binding of native LDL to the macrophage cell surface. By the same analysis, binding of ED-LDL to HMD-M was also saturable, specific, and involved a single class of binding sites. The B_{max} of ^{125}I-labeled ED-LDL was approximately 68 ng of LDL protein/mg of cell protein and the K_d was approximately 6.5×10^{-8} M.

Comparison of the B_{max} of native and ED-LDL shows that exactly double the quantity of ED-LDL binds to HMD-M. Since by way of competitive degradation studies, we have shown that ED-LDL enters the HMD-M via the LDL receptor, the binding results would imply that either twice as many LDL receptors were suddenly present on the surface of the HMD-M incubated at 4°C with ED-LDL or, much more likely, that ED-LDL was present as a dimer in the 4°C binding studies. If this were the case, we would expect the B_{max} of ED-LDL to be twice that of monomer-

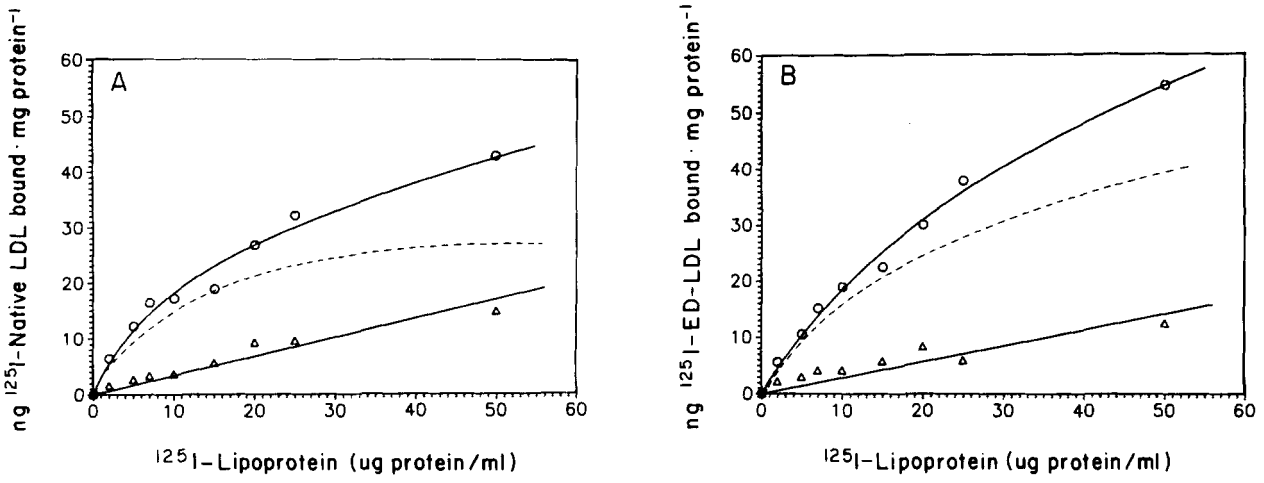

Fig. 8. Panel A: Binding of ^{125}I-labeled native LDL to HMD-M at 4°C. Monocytes were cultured for 14 days in DMEM (20% autologous serum). On day 14, the cells were washed with DMEM (10% ALPDS) and cooled to 4°C. The medium was removed and ^{125}I-labeled native LDL in DMEM (10% ALPDS) was added (at the concentrations shown on the abscissa) with and without a 30-fold protein excess of the corresponding unlabeled lipoprotein. Total binding (○) and nonspecific binding (△) are plotted. Each data point is the average of duplicate incubations. Panel B: Binding of ^{125}I-labeled ED-LDL to HMD-M at 4°C. Total binding (○) and nonspecific binding (△) are plotted. Each data point is the average of duplicate incubations. Subtraction of nonspecific binding curves from total binding curves represents specific binding (--).

ic native LDL when measured as μg of LDL protein/ml, since, in the case of the elastase-digested particles, every LDL particle bound to its receptor carries along with it an additional LDL particle. Likewise, a higher extracellular concentration of ^{125}I-labeled ED-LDL would be required to reach half-maximal binding to the cells (i.e., higher K_d), since 1 mole of native monomeric LDL would be equivalent to only 0.5 mol of dimerized ED-LDL when measured as μg of protein/ml. In order to verify the presence of LDL dimers, we performed the electrophoretic analyses reported below.

Analysis of lipoproteins by nondenaturing gradient gel electrophoresis (GGE)

Analysis by nondenaturing GGE (2.5–10%) revealed two bands for ED-LDL, a minor one migrating in the position of native LDL and a major one above it (**Fig. 9a,** lanes 2–4). The upper band in ED-LDL suggests that the modified particle was either larger than native LDL or had undergone a conformational change resulting in its anomalous mobility on the gel system used. Kallikrein-digested LDL in the same gel system exhibited only one band in the position of native LDL (data not shown).

In order to estimate the size of the upper band in ED-LDL, we cross-linked native LDL with dimethylsuberimidate and obtained several aggregated species (Fig. 9a, lane 5). The lowest M_r band in the cross-linked sample corresponded to that of native LDL. The next higher M_r band (presumably a dimer) migrated in approximately the same position as the upper band in the ED-LDL. A third cross-linked LDL species was visible at the top of the gel, and higher M_r aggregates of the cross-linked LDL did not enter the gel.

The introduction of excess negative charge on ED-LDL by succinylation or acetylation (Fig. 9b, lanes 8 and 9) resulted in the conversion of the upper band into the position of native LDL. The mobilities of acetylated and native LDL on this gel system were identical.

Taken together, these results are consistent with the 4°C binding data of Fig. 8b, suggesting that ED-LDL may exist as a dimer.

DISCUSSION

The results of our studies in which LDL digested by purified PMN elastase was incubated with HMD-M show that these LDL preparations exhibit a 1.5- to 2.5-fold greater binding and degradation by HMD-M than native LDL, and that this modified LDL also induces a greater stimulation of cholesterol esterification. We interpret our results to indicate that ED-LDL is taken up by the LDL receptor and not the scavenger receptor of HMD-M based on the following lines of evidence. *1*) Excess, unlabeled native LDL inhibited the macrophage degradation of ^{125}I-labeled ED-LDL by 90%; in the reverse experiment, unlabeled ED-LDL inhibited the degradation of ^{125}I-labeled native LDL by 90%; *2*) unlabeled acetylated LDL had the same minimal effect on the degradation of ^{125}I-labeled ED-LDL and ^{125}I-labeled native LDL; while unlabeled ED-LDL had no effect on the degradation of ^{125}I-labeled acetylated LDL; and *3*) the degradation of both ^{125}I-labeled na-

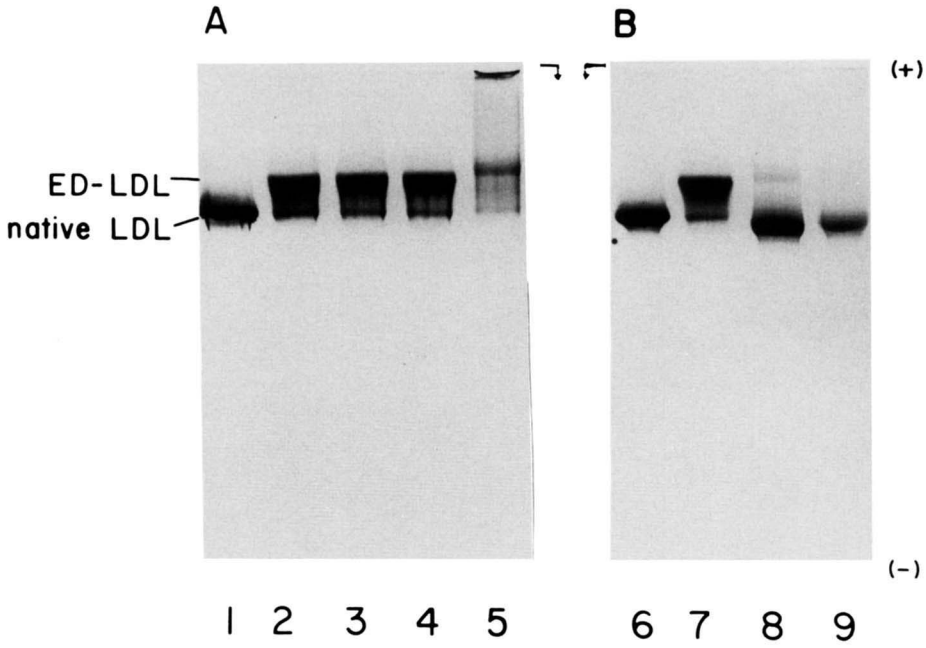

Fig. 9. Panel A: Nondenaturing GGE of native LDL, ED-LDL (15-, 60-, and 240-min digests) and chemically crosslinked LDL. Samples (15 μg of protein) were run on a 2.5–10% linear gradient gel (see Methods). Lane 1, native LDL; lane 2, ED_{15}-LDL; lane 3, ED_{60}-LDL; lane 4, ED_{240}-LDL; lane 5, native LDL that was crosslinked by incubation with dimethylsuberimidate (3:1, w/w, protein: crosslinker). See Methods for further details. Panel B: Nondenaturing GGE of native LDL, ED-LDL, succinylated ED-LDL, and acetylated ED-LDL. Succinylation and acetylation of ED-LDL were performed as described in Methods. Lane 6, native LDL; lane 7, ED-LDL; lane 8, succinylated ED-LDL; lane 9, acetylated ED-LDL.

tive LDL and ^{125}I-labeled ED-LDL was increased 2-fold by preincubating HMD-M in the presence of ALPDS.

Binding experiments at 4°C showed that at saturation, two-fold more ^{125}I-labeled ED-LDL bound to the macrophage cell surface than ^{125}I-labeled native LDL. Nondenaturing GGE revealed that ED-LDL migrated with an apparent M_r higher than that of native LDL. This apparently larger form of ED-LDL could be reversed to the position of monomeric native LDL by succinylation and/or acetylation. In addition, when ED-LDL was compared to a cross-linked sample of native LDL, ED-LDL migrated in the position of the next higher M_r band (presumably a dimer) above monomeric, native LDL. Due to the difficulties in establishing an M_r standard curve for macromolecules of this size ($3.0–9.0 \times 10^6$), we cannot state with certainty the molecular weights of the cross-linked LDL species. However, taken together, the 4°C binding data and the analyses of ED-LDL by nondenaturing GGE suggest that ED-LDL exists as a dimer. Further studies are needed to determine the exact physical state of ED-LDL.

While 4°C binding data (obtained at low extracellular concentrations of lipoprotein) shows the binding of ED-LDL to be saturable, the interaction of ED-LDL (at higher extracellular concentrations) with HMD-M at 37°C is obviously a more complex phenomenon, since degradation of ^{125}I-labeled ED-LDL at 37°C increases linearly from 20 to 300 μg of LDL protein/ml. A possible explanation is that, at high extracellular concentrations of ED-LDL, there may be an increase in the proportion of aggregated forms of the lipoprotein. In such a case the degradation curve for ED-LDL may saturate at concentrations higher than those tested in this study.

There are several reports in the literature on the receptor interactions of LDL after digestion with proteases. Chapman et al. (2) and Cardin et al. (3) showed that trypsin-digested LDL that had lost 20–25% of its surface-associated protein during proteolysis was degraded via the LDL receptor of human skin fibroblasts at a rate significantly lower than control LDL. Coetzee, Gevers, and van der Westhuyzen (4) digested LDL with plasmin under conditions where only 3% of the protein was hydrolyzed to TCA-soluble products. When this LDL was incubated with bovine smooth muscle cells in vitro, the rate of degradation was similar to that obtained when cells were incubated with native LDL. Yamamoto et al. (5) digested LDL with kallikrein and obtained four major fragments of apoB-100 with virtually no loss of protein. This modified LDL bound normally to human skin fibroblasts and stimulated cholesterol esterification in fibroblasts and MPM to the same degree as native LDL. From the above studies, it is clear that proteolysis of apoB-100 per se is insufficient to alter the recognition of LDL by the LDL receptor. Only

trypsin-treated LDL, where up to 25% of the surface protein was lost, exhibited a significant reduction in binding and degradation by fibroblasts in culture. Consequently, it appears that the effect of PMN elastase on LDL apoB-100 causing enhanced degradation of the lipoprotein particle by HMD-M via the LDL receptor is unique.

Various chemical modifications of LDL such as acetylation, acetoacetylation, and reaction with malondialdehyde have been shown to enhance uptake and degradation of the lipoproteins by macrophages. However, in contrast to ED-LDL, these chemically modified preparations are taken up via the macrophage scavenger receptor, unique in its high affinity for negatively charged ligands and its lack of regulation by intracellular cholesterol concentrations.

There have been numerous reports indicating that oxidized LDL is degraded more readily by macrophages than native LDL and that uptake occurs via the scavenger receptor (26–28). Raymond and Reynolds (29) isolated LDL from interstitial inflammatory fluid of the rabbit employing the sponge-implantation model of inflammation. The LDL isolated from this fluid exhibited altered composition, enhanced anodal mobility on agarose gel electrophoresis, and no evidence of lipid peroxidation as measured by the thiobarbituric acid assay. We suspect that LDL modification was due, at least in part, to proteolysis of apoB-100 by enzymes liberated from activated PMN. When this LDL was incubated for 24 hr with MPM, it was degraded threefold faster than native plasma LDL, presumably via the scavenger receptor since its uptake was inhibited by the presence of excess acetylated LDL (30). However, it should be noted that the authors used Ham's F-10 medium in their macrophage incubations which contains relatively high levels of iron and copper; Parthasarathy et al. (26) have shown that LDL undergoes oxidation when incubated with MPM in serum-free Ham's F-10 medium, and suggested that the iron and copper were responsible for promoting the transition metal-catalyzed oxidation of LDL. Thus it is possible that the LDL isolated from inflammatory fluid by Raymond and Reynolds (29) were first modified by proteolysis and further altered by oxidation during incubation with MPM with consequent recognition of the modified LDL by the macrophage scavenger receptor. In accord with this interpretation are the recent findings by the same authors using a system in which ^{125}I-labeled native LDL was incubated with a mixture of cells containing 89% PMN and 9% monocyte-macrophages, isolated from the inflammatory fluid of the sponge-implanted rabbit (10). The modified LDL showed on SDS-PAGE numerous bands of M_r lower than apoB-100 and exhibited an "apparent" increase in cell surface binding by macrophages. Although oxidation was considered to be at the basis of the phenomenon, we would like to suggest that an important proteolytic component was also present, namely that of PMN elastase. This is based on the following considerations: *1*) the inflammatory fluid LDL and ED-LDL exhibited degradation of apoB-100 on SDS-PAGE; *2*) the reisolated inflammatory fluid LDL did not appear to be oxidized as judged by its lack of reactivity in the thiobarbituric acid assay; and *3*) inflammatory cell-modified LDL and ED-LDL had a binding behavior similar to macrophages and both had an apparent increased particle size.

Our studies using ED-LDL and HMD-M cultured in DMEM help to sort out two of the factors by which activated PMN or HMD-M cause LDL modification and influence their subsequent metabolic fate. By using purified PMN elastase, one eliminates the possibility of PMN-mediated oxidation of LDL, and by using HMD-M cultured in DMEM, a medium which contains no copper and little iron, transition metal-catalyzed oxidative events are precluded.

The in vivo significance of LDL oxidation, proteolysis, or a combination of both in the enhanced uptake of LDL-derived cholesterol by macrophages remains to be determined. Both oxidation and proteolysis can act synergistically and this possibility is suggested by the data of Davies (31) indicating that oxidized proteins are more susceptible to proteolysis at neutral pH. The importance of our studies is that they show that a specific proteolytic modification alone can lead to the enhanced uptake of LDL by HMD-M, and that this occurs via the LDL receptor rather than the scavenger receptor. The type of proteolytic LDL modification we have described could occur in vivo in the presence of anti-oxidants at inflammatory sites rich in activated PMN, in a microenvironment wherein high M_r protease inhibitors might be either physically excluded, saturated, or inactivated by proteolysis and/or oxidation. One could speculate that the early fatty streak represents a special form of an inflammatory response. In such a case, the proteolytic modification of LDL by PMN elastase may be relevant to the atherosclerotic process.

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