Fusion of Proteolyzed Low-Density Lipoprotein in the Fluid Phase: A Novel Mechanism Generating Atherogenic Lipoprotein Particles

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ABSTRACT: During atherogenesis, lipid droplets appear in the extracellular space of the arterial intima. We previously observed generation of lipid droplets on the surface of exocytosed mast cell granules when granule neutral proteases degraded the granule-bound LDL particles and the particles became unstable and fused [Kovanen, P. T., & Kokkonen, J. O. (1991) *J. Biol. Chem. 266*, 4430−4436]. We have now extended our studies to the fluid phase and examined the effects of several proteases (trypsin, α-chymotrypsin, Pronase, plasmin, kallikrein, and thrombin) all known for their ability to cleave the apolipoprotein B-100 component (apoB-100) of LDL. The fused LDL particles were separated from unfused particles by gel filtration or by density gradient ultracentrifugation. Proteolytic degradation of LDL with trypsin, α-chymotrypsin, or Pronase led to fragmentation of apoB-100 and release of the fragments from the LDL particles and triggered particle fusion. In contrast, proteolytic degradation of LDL with plasmin, kallikrein, or thrombin, which also led to fragmentation of apoB-100 but not to release of fragments, did not trigger particle fusion. With advancing degradation of apoB-100, particles having progressively lower densities and larger sizes were generated. Thus, after incubation for 24 h with α-chymotrypsin (apoB-100:α-chymotrypsin mass ratio 10:1) 40% of the apoB-100 was degraded and about 30% of the LDL particles had fused and reached diameters of up to 70 nm and densities ranging from 1.020 to <1.005 g/mL. When the proteolyzed LDL particles, both unfused and fused, were incubated with macrophages, only those particles that had undergone fusion were ingested and converted into intracellular cholesteryl ester droplets. Thus proteolysis of LDL with release of apoB-100 fragments renders the particles sufficiently unstable to fuse and thus to become liable to ingestion by macrophages. Since the fused LDL particles resemble the extracellular lipid droplets in the atherosclerotic arterial intima and generate foam cells in vitro, these findings support the idea that proteolytic fusion of LDL is an atherogenic process.

Atherosclerosis is a disease characterized by accumulation of cholesterol in the inner layer of the arterial wall, the arterial intima (Stary et al., 1992; Smith, 1974). Cholesterol, which is thought to originate from plasma low-density lipoprotein (LDL),[1] accumulates both intracellularly and extracellularly in the form of lipid droplets. The molecular mechanisms leading to the formation of intracellular lipid droplets in macrophages are well characterized (Brown & Goldstein, 1983). Less research has focused on the mechanisms by which extracellular lipid droplets are generated in the arterial intima in vivo.

At the light microscopic level, accumulation of extracellular cholesterol occurs initially as fine perifibrous droplets either in the subendothelial proteoglycan-rich layer (Pasquinelli et al., 1989), or in the deep elastin-rich layer of the arterial intima (Guyton et al., 1990). Several lines of evidence suggest that most of the extracellular droplets are derived directly from LDL particles rather than from intracellular droplets released, for instance, from dying foam cells. Thus, chemical analysis of the lipids present in intimal areas that contain perifibrous lipid droplets (prelesional areas) disclosed that the fatty acid composition of cholesteryl esters is virtually identical with that of LDL rather than of foam cells, the majority of the cholesterol molecules being esterified to linoleate (Smith, 1974). This contrasts sharply with the fatty acid composition of intracellular lipid droplets, in which the major fatty acid esterified to cholesterol is oleate (Smith, 1974; Brown et al., 1980). Furthermore, electron microscopic studies of extracellular lipid droplets have shown them to be of the size range 30−400 nm, a finding also compatible with the idea that the droplets are derived from native LDL particles rather than from intracellular lipid droplets, the former being smaller (20−25 nm) and the latter larger (400−6000 nm) than the perifibrous lipid droplets (Guyton et al., 1990).

On incubating LDL particles with exocytosed rat mast cell granules, i.e., granule remnants, we observed formation of lipid droplets with diameters ranging from 30 to 100 nm, i.e., falling within the range of the intimal extracellular lipid droplets (Kovanen & Kokkonen, 1991). Thus, when incubated with granule remnants, LDL particles bind to the heparin proteoglycan component of the remnants, whereupon the two neutral proteases of the remnants, chymase and carboxypeptidase A, degrade the apoB-100 of the heparin-bound LDL particles, so rendering the particles unstable and triggering their fusion. These studies suggested that proteolytic modification of LDL was one possible mechanism for the generation of the extracellular lipid droplets. In the present study, we extend our research on proteolytic modi-

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[1] Abbreviations: apo, apolipoprotein; CL, cholesteryl linoleate; LDL, low-density lipoproteins.

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fication of LDL to investigating the effect of various proteolytic enzymes on LDL particle size in the fluid phase. We now report that mere fragmentation of apoB-100 does not destabilize the particles sufficiently to induce their fusion. Fusion occurs only when fragmentation of the apoB-100 of LDL is followed by release of fragments.

EXPERIMENTAL PROCEDURES

Materials. α-Chymotrypsin, bovine serum albumin, kallikrein, plasmin, Pronase E, thrombin, trypsin, and soybean trypsin inhibitor were from Sigma; [1,2-^{3}H]cholesteryl linoleate, *tert*-butoxycarbonyl-L-[^{35}S]methionine *N*-hydroxysuccinimidyl ester (^{35}S-labeling reagent) and [1-^{14}C]oleic acid were from Amersham; Celite 545 (acid washed) was from Fluka; FastLane agarose was from FMC BioProducts; Eagle's basal medium with Earle's salts and 20 mM Hepes (EBME) were from Flow Laboratories; Dulbecco's phosphate-buffered saline (PBS), RPMI 1640 culture medium with 25 mM *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid, fetal calf serum, penicillin, and streptomycin were from GIBCO; Superose HR 10/30 columns were from Pharmacia LKB Biotechnology; and female NMRI mice (20−30 g) were obtained from a licensed animal center (Poikkijoki, Kuvaskangas, Finland). Semipurified (60−70% purity) cholesteryl ester transfer protein was a kind gift from Drs. C. Ehnholm and M. Jauhiainen, National Public Health Institute, Helsinki, Finland.

Preparation and Labeling of LDL. Human LDL (d = 1.019−1.050) was isolated from plasma of healthy volunteers by sequential ultracentrifugation in the presence of 3 mM Na_2EDTA (Havel et al., 1955; Radding & Steinberg, 1960). [^{3}H]Cholesteryl linoleate was incorporated into LDL by incubating LDL with solid dispersions of [^{3}H]cholesteryl linoleate on acid-washed Celite 545 essentially as described in Kovanen and Kokkonen (1991), except that cholesteryl ester transfer protein and isolated LDL were used instead of serum. For this purpose, 200 μCi of [^{3}H]cholesteryl linoleate in chloroform was adsorbed onto 300 mg of the Celite in a 25-mL conical glass tube under a gentle stream of nitrogen. To the Celite were added 5 mg of LDL and 0.2−0.5 mg of cholesteryl ester transfer protein (activity 5−10 μmol of cholesteryl ester transferred mg^{-1} h^{-1}) in 2 mL of buffer A (150 mM NaCl, 1 mM EDTA, 5 mM Tris hydrochloride, pH 7.4). The mixture was incubated at 37 °C for 18−24 h with rotation in a Coulter mixer. After incubation, the Celite was sedimented by centrifugation at 1500g for 15 min. The supernatant containing LDL was centrifuged at 12000g for 5 min and run through a Bio-Gel A-5m column (1 × 40 cm) equilibrated in buffer A. Chromatography was performed at 7 mL/h, and the fractions containing the labeled LDL were pooled. The specific radioactivities of the [^{3}H]-CE-LDL preparations were 22−44 dpm/ng of LDL protein. To obtain ^{35}S-apoB-100-LDL, the protein component of LDL, apoB-100, was labeled by the Bolton−Hunter procedure (Bolton & Hunter, 1973) with a ^{35}S-labeling reagent, as previously described (Kovanen & Kokkonen, 1991). The specific radioactivities of the ^{35}S-LDL-apoB-100 preparations were 11−22 dpm/ng of LDL protein. For each experiment, labeled LDL was diluted with unlabeled LDL to give the specific radioactivities indicated in the figure legends. The concentration of LDL is expressed in the text in terms of its protein concentration.

Proteolytic Degradation of LDL and Isolation of Fused LDL Particles. The standard degradation assay was conducted at 37 °C in 100−200 μL of buffer A containing 1.0 mg/mL LDL, [^{3}H]CE-LDL, or a mixture of [^{3}H]CE-LDL and ^{35}S-apoB-100-LDL and 0.1 mg/mL α-chymotrypsin. In every experiment, unlabeled LDL and both ^{3}H-labeled and ^{35}S-labeled LDL were derived from the same batch of LDL. In one experiment (Figure 5), LDL was incubated not only with α-chymotrypsin but also with Pronase, trypsin, kallikrein, plasmin, or thrombin and with a mixture containing kallikrein, plasmin, and thrombin. After each incubation, the degree of proteolytic degradation of ^{35}S-apoB-100-LDL was determined from a sample of the incubation mixture. For this purpose, 80 μL of ice-cold buffer A containing 5 mg/mL bovine serum albumin and 25 μL of 50% (w/v) trichloroacetic acid was added to 20-μL samples of the incubation mixtures. After incubation for 30 min at 0 °C, the mixtures were centrifuged at 12000g for 10 min, 100-μL samples of the supernatants were taken, and their ^{35}S radioactivities were determined. The degree of apoB-100 degradation is expressed as the amount of trichloroacetic acid-soluble radioactivity produced. Blank values were obtained by precipitating untreated LDL with trichloroacetic acid.

Gel filtration was used to separate fused LDL particles from native LDL. The samples corresponding to 100−200 μg of undegraded LDL were run in buffer A through two Superose HR 10/30 columns connected in series. The flow rate was 500 μL/min, and fractions of 500 μL were collected. The elution of the samples was monitored at 280 nm. To determine the degree of fusion of [^{3}H]CE-LDL, the ^{3}H radioactivity of fractions 30−60 was measured (the total radioactivity eluted), and the ratio of the radioactivity in fractions 30−40 (void volume peak) to the total radioactivity eluted was calculated.

Analysis of Proteolyzed LDL Samples by SDS−PAGE and by FastLane Agarose Gel Electrophoresis. After incubation of LDL with the various proteases, 10−20-μL samples corresponding to 10−20 μg of undegraded LDL and 5-μL samples containing 5 μg of untreated LDL were run on Bio-Rad 4−20% sodium dodecyl sulfate−polyacrylamide gels (SDS−PAGE) using the Laemmli buffer system under reducing conditions (Laemmli, 1970). The gels were stained with 0.1% Coomassie Brilliant Blue, destained with 40% (v/v) methanol−10% (v/v) acetic acid, and photographed.

For FastLane agarose gel electrophoresis, 10-μL aliquots of the samples corresponding to 10 μg of undegraded LDL were mixed with 10 μL of 0.5% Sudan black in ethylene glycol and 3 μL of glycerol. The samples were then run on a 0.5% FastLane agarose gel in 50 mM Tris barbital−sodium barbital buffer, pH 8.8, and photographed.

Equilibrium Density Gradient Centrifugation of Proteolyzed LDL. [^{3}H]CE-LDL (200 μg) in 200 μL of buffer A was incubated at 37 °C for 12, 24, or 72 h with 20 μg of α-chymotrypsin or incubated at 37 °C for 72 h in the absence of α-chymotrypsin. Buffer A (800 μL) was added to each LDL preparation, and the samples were layered on top of an 11-mL sucrose gradient. The sucrose gradient was prepared by freezing 11 mL of 7% (w/v) sucrose in aqueous solution containing 1 mM EDTA and thawing the solution at 4 °C. This treatment yielded a linear sucrose gradient with densities ranging from 1.002 to 1.072 g/mL. The samples were centrifuged in a Beckman SW 40 Ti rotor at 250000g at 15 °C for 72 h, and then 400-μL fractions were collected, starting from the top of the gradient, and their

radioactivities were counted. The density of each fraction was determined by refractometry.

Isolation of Mouse Peritoneal Macrophages. Macrophages were harvested from unstimulated NMRI mice in Dulbecco's phosphate-buffered saline containing 1 mg/mL bovine serum albumin (Goldstein et al., 1979). The peritoneal cells were resuspended in RPMI 1640 culture medium containing 20% heat-inactivated fetal calf serum, 100 IU/mL penicillin, and 100 μg/mL streptomycin. Cells ($1-2 \times 10^6$) were seeded into plastic 24-well plates (Falcon) and incubated overnight at 37 °C in a humidified CO_2 (5% CO_2 in air) incubator. Before the experiments, the dishes were washed three times with 500 μL of EBME medium to remove nonadherent cells. For cholesteryl oleate mass determination (Table 1), 2.5×10^6 cells were seeded into 12-well plates.

Preparation of Fused LDL for Cell Culture Experiments. [^{3}H]CE-LDL (8 mg) and α-chymotrypsin (800 μg) were incubated for 72 h at 37 °C under sterile conditions in 1.5 mL of buffer A containing 100 IU/mL penicillin and 100 μg/mL streptomycin. After incubation, the NaCl concentration of the sample was increased from 150 to 250 mM, and the sample was divided into two fractions. Both fractions were layered beneath 11 mL of buffer A and centrifuged for 20 h at 4 °C in a Beckman SW 40 Ti rotor at 220000*g*. After centrifugation, the floating material was collected.

For determination of the macrophage cholesteryl oleate mass (Table 1), 1 mg of [^{3}H]CE-LDL (170 dpm/μg) was proteolyzed with either 100 μg of α-chymotrypsin, 50 millunits of plasmin, 50 millunits of kallikrein, or 20 units of thrombin or a mixture of plasmin (12 millunits), kallikrein (18 millunits), and thrombin (6 units) in 1 mL of buffer A at 37 °C for 40 h. After incubation, each mixture was layered on top of a linear sucrose gradient ($d = 1.002-1.072$ g/mL) and centrifuged at 15 °C for 20 h in a Beckman SW 40 Ti rotor at 250000*g*. Fractions of 800 μL were collected and their radioactivities were measured. The peak fractions (according to radioactivity) of the variously treated LDL particles were pooled and concentrated by ultrafiltration, and samples containing 75 μg of cholesteryl linoleate were added to the macrophage monolayers in 600 μL of medium.The pools were as follows: untreated LDL, fractions 6−8 ($d = 1.026$); plasmin-treated LDL, fractions 7−9 ($d = 1.029$); kallikrein-treated LDL, fractions 8−10 ($d = 1.031$); thrombin-treated LDL, fractions 8−10 ($d = 1.029$); LDL treated with a mixture of all three above enzymes, fractions 7−9 ($d = 1.032$); α-chymotrypsin-treated LDL, fraction 1 ($d = 1.005$, fused LDL).

Incorporation of Oleate into Cholesteryl Oleate by Macrophages. Each monolayer of macrophages received 500 μl of EBME medium containing 10 mg/mL bovine serum albumin, 100 IU/mL penicillin, 200 μM [^{14}C]oleate−albumin, and 125 μg of LDL (native or fused) as cholesteryl linoleate. After incubation at 37 °C for the indicated times, the monolayers were washed with 2 × 500 μL of PBS, and the lipids were extracted in situ with hexane/isopropyl alcohol (3:2). The cholesteryl [^{14}C]oleate was isolated by silica gel thin-layer chromatography. The cholesteryl oleate band was visualized with iodine vapor, cut off and tranferred into a vial, and counted in a Beckman scintillation counter (Brown et al., 1980). The cells in the monolayers were then dissolved in 0.2 M NaOH, and aliquots were removed for determination of protein. For determination of the cholesteryl oleate mass, each monolayer of macrophages received 600 μL of EBME medium containing 10 mg/mL of bovine serum albumin, 100 IU/mL penicillin, 100 μg/mL streptomycin, 200 μM oleate−albumin, and 75 μg of LDL (native or fused) as cholesteryl linoleate. After incubation at 37 °C for 24 h, the monolayers were washed with PBS and the cells were dissolved in 0.2 M NaOH. A portion was removed for protein determination, and the rest of the sample was extracted overnight in chloroform−methanol (2:1) at 4 °C. The organic phase was removed and the aqueous phase was rinsed with chloroform. Organic phases were combined and stored −20 °C.

Determination of Cholesteryl Oleate Mass. Extracted chloroform phases were evaporated to dryness with a stream of N_2 and then dissolved in 100 μL of the elution sovent. Determination was perfomed by reversed-phase HPLC on a 0.3 × 25 cm Spherisorb S5 ODS2 column by isocratic elution with acetonitrile−isopropyl alcohol (30:70) at a flow rate of 300 μl/min, and lipids were detected by UV absorbance at 210 nm (Kritharides et al., 1993). Cholesteryl heptadecanoate (0.5−1.0 μg) was used as internal standard. The chromatographic apparatus consisted of an Applied Biosystems 400 solvent delivery system, a 783 programmable absorbance detector, and a Hewlett-Packard 3396A integrator.

Oil Red O Staining of Lipid Droplets in Macrophages. Macrophages were isolated as described above, except that cells were plated on 13-mm glass coverslips within 24 wells. Each monolayer of macrophages received 500 μL of EBME medium containig 10 mg/mL bovine serum albumin, 100 IU/mL penicillin, and 125 μg of LDL (native or fused) as cholesteryl linoleate. After incubation at 37 °C for 24 h, the cells were washed with 2 × 500 μL of PBS, fixed with 300 μL of 4% formaldehyde−0.05% glutaraldehyde, stained with 300 μL of 0.5% Oil Red O, and counterstained with 300 μL of Harris hematoxylin. The coverslips were mounted on glass microscope slides with 5% gelatin−50% glycerol and photographed (Humason, 1979).

Electron Microscopic Examination of the LDL Fractions. Samples were mixed 1:1 with 1% potassium phosphotungstate, pH 7.4, and the mixtures were dried on carbon-coated grids (Forte & Nichols, 1972). Negatively stained samples were viewed and photographed in a Jeol 100CX electron microscope at the Department of Electron Microscopy, University of Helsinki. The diameters of 250 randomly selected lipoprotein particles were measured from the electron micrographs of each LDL fraction.

Other Assays. Protein was determined by the method of Lowry et al. (1951), with bovine serum albumin as standard.

RESULTS

To study the effect of proteolytic degradation of LDL particles on their size distribution, 1 mg/mL LDL was incubated with 0.1 mg/mL α-chymotrypsin. α-Chymotrypsin has a specificity close to that of mast cell chymase, a neutral protease which was previously shown to trigger LDL fusion (Kovanen & Kokkonen, 1991). After incubation at 37 °C for 24 h, aliquots of the incubation mixtures were subjected to polyacrylamide gel electrophoresis in the presence of SDS. Figure 1A shows that α-chymotrypsin had produced extensive fragmentation of apoB-100, reflected by complete disappearance of the apoB-100 band. A parallel experiment was conducted with LDL, in which the apoB-100 component had been labeled with ^{35}S (^{35}S-apoB-100-LDL). Determination of the production of ^{35}S-labeled

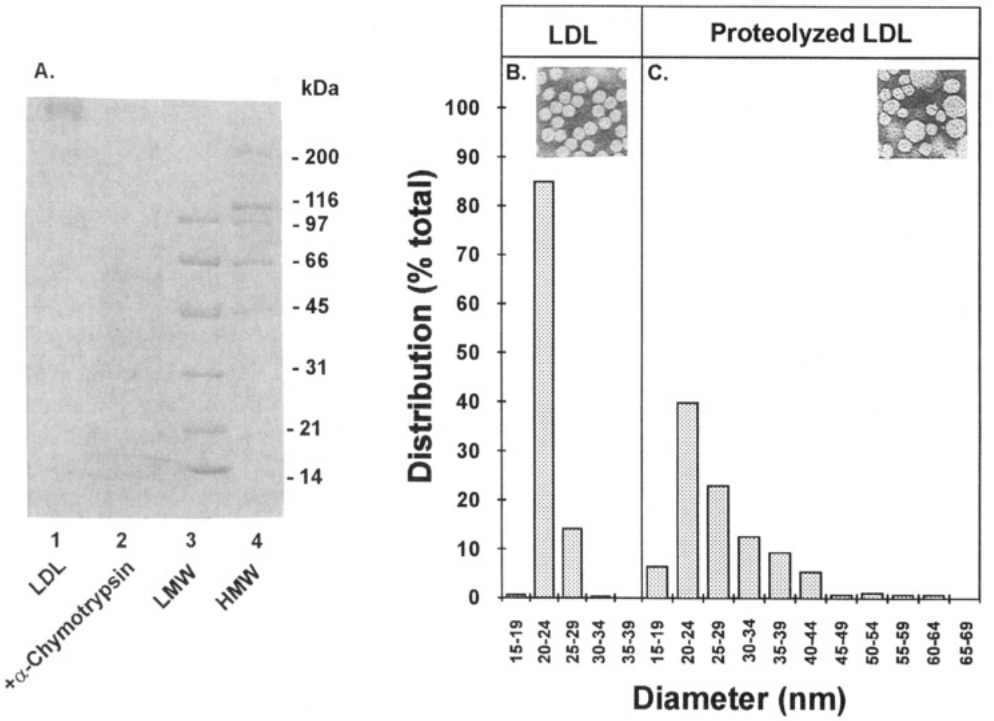


FIGURE 1: SDS−PAGE analysis of apoB-100 and electron microscopic analysis of LDL particles incubated with α-chymotrypsin. LDL (200 μg) was incubated in 200 μL of buffer A in the absence (lane 1 in panel A; panel B) or presence (lane 2 in panel A; panel C) of 20 μg of α-chymotrypsin at 37 °C for 24 h. After incubation, the mixtures were prepared for SDS−PAGE analysis and for electron microscopy. The distribution of the diameters of the negatively stained LDL particles (insets) was determined from electron micrographs as described under Experimental Procedures. LMW = low-molecular-weight standards; HMW = high-molecular-weight standards.

trichloroacetic acid-soluble degradation products revealed that incubation of the ^{35}S-apoB-100-LDL with α-chymotrypsin under the conditions defined above led to 40% degradation of the labeled apoB-100. Samples of native and proteolyzed LDL were prepared for negative staining electron microscopy, and the size distribution of the particles was determined. As shown in Figure 1, the proteolyzed particles (panel C) were, on average, larger, and their size distribution was more heterogeneous than that of LDL particles incubated in the absence of α-chymotrypsin (panel B). Thus, the mean diameter of the protease-treated LDL particles was 28 nm (±7.8 nm; median 26 nm; Figure 1C) whereas that of the control particles was 24 nm (±2.0 nm, SD; median 23 nm; Figure 1B) which, again, resembled that of native untreated LDL (mean 23 ± 2.0 nm; median 23 nm; not shown).

We next used gel filtration on Superose 6HR 10/30 columns to separate particles of different sizes. For this purpose, LDL was incubated for 24 h in the absence or presence of α-chymotrypsin (LDL:α-chymotrypsin mass ratio 10:1), and the incubation mixtures were run through a system consisting of two Superose 6HR 10/30 columns connected in series. As shown in Figure 2 (panel A), the elution profile of the control LDL showed one symmetrical peak. In contrast, the elution profile of the proteolyzed LDL showed two peaks (panel B): peak I material emerged in a position (fractions 30−40) corresponding to the void volume of the column (fraction 30), and peak II material (fractions 41−60) eluted in a position roughly corresponding to the nonproteolyzed native LDL. The peak fractions of peak I and peak II were prepared for electron microscopy, and the size distributions of the particles contained in these fractions were analyzed with the technique of negative staining. It appeared that the majority of the particles in peak I (mean 37 ± 8.5 nm; median 35 nm; panel D) were larger than those in peak II (mean 24 ± 3 nm; median 23 nm; panel E) which had a size distribution similar to that of the nonproteolyzed LDL particles (mean 23 ± 2.0 nm; median 23 nm; panel C). This result showed that the proteolyzed and fused LDL particles can be separated from the proteolyzed but unfused LDL particles, and moreover it confirmed that prolonged treatment of the LDL with α-chymotrypsin leads to an increase in the size of the LDL particles.

The above method of isolation of fused LDL particles allowed us to design experiments in which the degree of LDL fusion was studied. For this purpose, cholesteryl linoleate, i.e., the main component of the LDL core, was radiolabeled with ^{3}H. The ratio of the ^{3}H radioactivity in peak I (fractions 30−40) containing the fused LDL to the total eluted ^{3}H radioactivity is used as a measure of the degree of LDL fusion. In a preliminary experiment we observed that after 2, 4, 6, and 8 h of incubation, about 1%, 6%, 8%, and 11% of LDL became fused. LDL was then incubated with α-chymotrypsin for various lengths of time, and the degree of LDL fusion and of apoB-100 degradation was determined at various time intervals. Figure 3A shows that the degree of fusion of LDL increased progressively so that, after incubation for 72 h, more than half of the LDL particles had fused to form large particles. Figure 3B (inset) shows that, during incubation, LDL was also progressively proteolyzed so that, by the end of the incubation period, up to 60% of the ^{35}S-apoB-100 had been degraded into trichloroacetic acid-soluble material. The degree of fusion also depended on the LDL concentration used. Thus, incubation of 0.1, 0.3, 0.5, 1, 2, 3, and 5 mg/mL LDL with 0.1 mg/mL α-chymotrypsin for 24 h resulted in increasing degrees of fusion, amounting to 22%, 26%, 27%, 31%, 34%, 35%, and 37%, respectively (not shown).

Fused LDL and native-sized LDL could also be separated by agarose gel electrophoresis using large-pore agarose designed for separation of megabase DNA (FastLane aga-

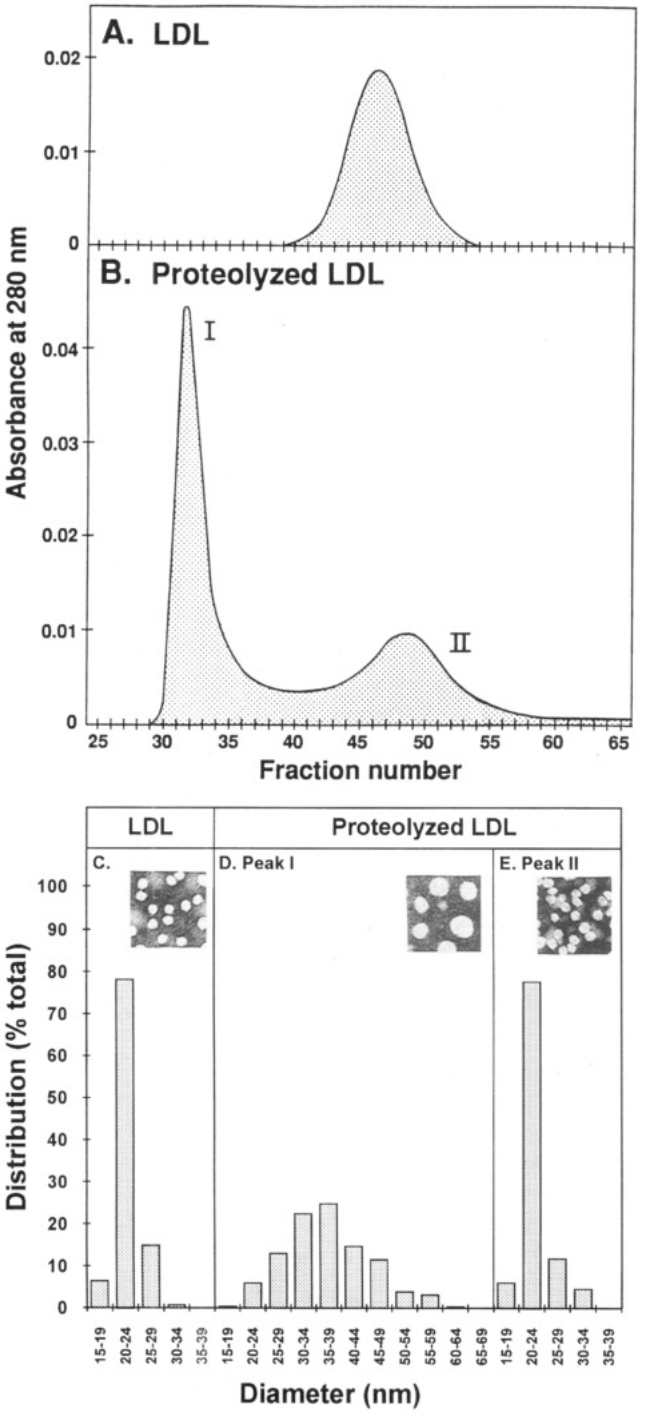


FIGURE 2: Analysis of α-chymotrypsin-treated LDL by gel filtration. LDL (200 μg) was incubated in 200 μL of buffer A in the absence (panels A and C) or presence (panels B, D, and E) of 20 μg of α-chymotrypsin at 37 °C for 24 h. After incubation, the mixtures were applied to a gel filtration system consisting of two Superose 6 HR 10/30 columns connected in series and eluted with buffer A at a flow rate of 0.5 mL/min at 4 °C, and fractions of 500 μL were collected. The elution profiles were monitored at 280 nm. The peak fractions were prepared for electron microscopy (insets), and the distribution of the diameters of the negatively stained LDL particles was determined from electron micrographs as described under Experimental Procedures.

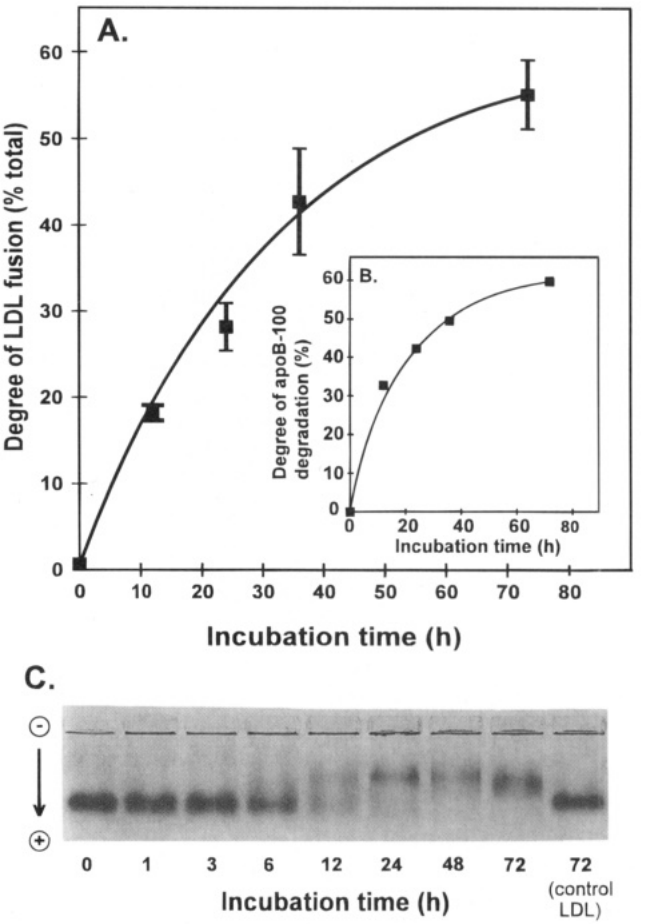


FIGURE 3: Superose gel filtration and FastLane agarose electrophoresis of LDL incubated for different times with α-chymotrypsin. (A) [^{3}H]CE-LDL (200 μg) (2500 dpm/μg) was incubated in 200 μL of buffer A in the presence of 20 μg of α-chymotrypsin at 37 °C for the indicated time periods. After incubation, the mixtures were applied to Superose 6 HR 10/30 columns and eluted with buffer A (at a flow rate of 0.5 mL/min at 4 °C), the 500-μL fractions were collected, and their radioactivities were counted (see Figure 2). The degree of fusion is expressed in percent and was calculated by dividing the quantity of ^{3}H radioactivity in fractions 30−40 by the total quantity of radioactivity eluted (fractions 30−60) (see Figure 2A,B). The average recoveries of fused and unfused LDL particles were 60% and 75%, respectively. In control incubations, [^{3}H]CE-LDL was incubated in the absence of α-chymotrypsin, and the values were subtracted from the corresponding values obtained in the presence of α-chymotrypsin. At each time point, the mean ± SD (n = 3) is shown. (B) ^{35}S-apoB-100-LDL (200 μg) (1200 dpm/μg) was incubated as in (A). After the indicated time periods, the degree of proteolytic degradation of ^{35}S-apoB-100 was determined by measuring the quantity of ^{35}S-labeled trichloroacetic acid-soluble products as described in Experimental Procedures. (C) LDL (200 μg) was incubated at 37 °C in 200 μL of buffer A in the presence or absence of 20 μg of α-chymotrypsin for the indicated time periods. Then 10-μL aliquots of the incubation media, corresponding to 10 μg of undegraded LDL, were taken at the end of incubation, mixed with 10 μL of Sudan black, and run on a 0.5% FastLane agarose gel in 50 mM barbital buffer, pH 8.8.

rose). LDL was incubated with α-chymotrypsin (mass ratio 10:1) for various lengths of time, and aliquots of the incubation mixtures were run on 0.5% FastLane agarose. In this electrophoretic system, control (native) LDL appeared as a single band (Figure 3C). During incubation, the electrophoretic mobility of a fraction of the LDL became increasingly retarded, as reflected in broadening of the band (at 3 h) and ultimately in formation of a second band (at 6 h). With prolongation of the incubation time up to 72 h, the intensity of the second band increased and that of the LDL band decreased. To evaluate whether apoB-100 degradation possibly contributed to the retarded electrophoretic mobility of proteolyzed LDL, we incubated LDL with α-chymotrypsin at 15 °C for 72 h, a temperature which permits apoB-100 degradation (at low speed) but prevents LDL fusion (Paananen & Kovanen, 1994). During the

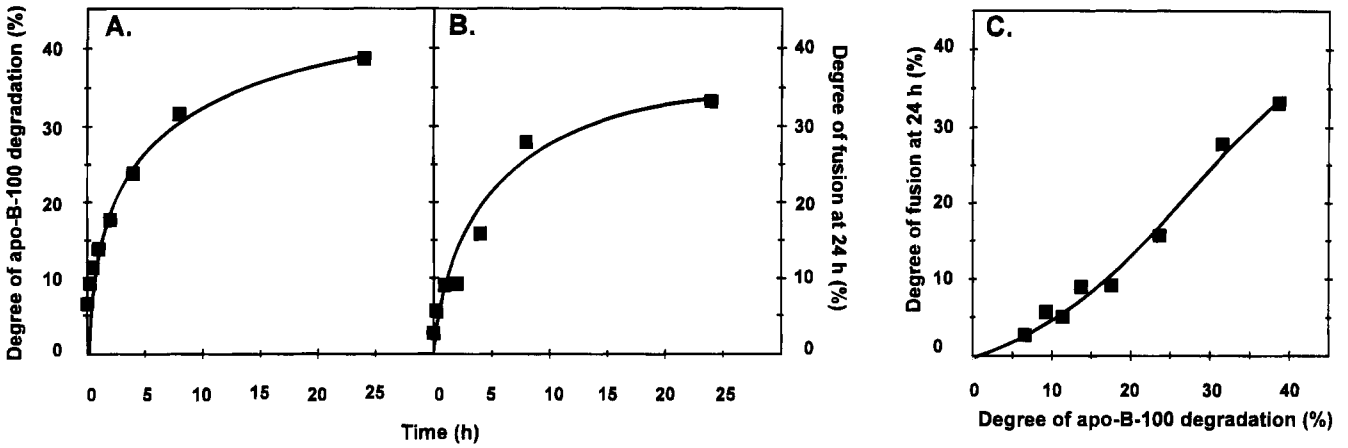


FIGURE 4: Effect of the degree of apoB-100 degradation on the degree of LDL fusion. LDL (220 μg) containing both [^{3}H]CE-LDL (2500 dpm/μg of cholesteryl linoleate) and ^{35}S-apoB-100-LDL (1200 dpm/μg of protein) was incubated in 220 μL of buffer A in the presence of 22 μg of α-chymotrypsin at 37 °C for 24 h. At the time points indicated (panel A, 0, 15, and 30 min and 1, 2, 4, 8, and 24 h), 200 μg of trypsin inhibitor was added to the reaction mixtures, and samples were taken for determination of the degree of proteolytic degradation of LDL (A). This was determined by measuring the quantity of ^{35}S-labeled trichloroacetic acid-soluble products. At the end of incubation (at 24 h), the degree of LDL fusion was determined by Superose gel filtration (B). The degree of fusion during the 24-h incubation period is plotted as a function of the degree of apoB-100 degradation (C).

incubation, up to 17% of the apoB-100 present was degraded. It appeared that the proteolyzed but nonfused LDL moved slightly faster than native LDL (data not shown).

In the above experiments, progressing degradation of apoB-100 was accompanied with increasing fusion of LDL. Hence, it was impossible to tell whether the fusion process depended on the degree of apoB-100 degradation. To selectively prevent apoB-100 degradation, trypsin inhibitor was added to the incubation mixtures. For this purpose, LDL was incubated with α-chymotrypsin for 24 h. Trypsin inhibitor was added to the incubation mixtures at various time points (0, 15, or 30 min or 1, 2, 4, 8, or 24 h) after the start of incubation. The generation of trichloroacetic acid-soluble degradation products was measured for each initial period without trypsin inhibitor, and the degree of LDL fusion was measured at the end of each incubation (at 24 h). ApoB-100 degradation increased initially rapidly and then more slowly to reach a level of 38% (Figure 4A). The degree of LDL fusion also increased progressively so that 33% of the LDL particles had fused when apoB-100 degradation was allowed to proceed throughout the 24-h incubation period (Figure 4B). When the degree of apoB-100 degradation was related to the degree of LDL fusion (Figure 4C), it appeared that the rate of fusion initially tended to be slow. Not until about 15% of apoB-100 had been degraded did the rate of fusion begin to accelerate slightly. The above results showed that, within the range of apoB-100 degradation studied (5−38%), there was no threshold for LDL fusion.

To extend the repertoire of proteases used for induction of LDL fusion, we included in the study three enzymes known specifically to cleave the apoB-100 of LDL, plasmin, kallikrein, and thrombin (Cardin et al., 1984; Coetzee et al., 1980). In addition, LDL was incubated with trypsin and Pronase, two enzymes which, like α-chymotrypsin, degrade apoB-100 extensively. After incubation of LDL with these enzymes, the extent of LDL fusion and the quantity of ^{35}S-labeled trichloroacetic acid-soluble degradation products released were determined (Figure 5A). Furthermore, the degree and patterns of apoB-100 fragmentation in the LDL particles after treatment with each of these enzymes were determined (Figure 5B). It appeared that, during incubation of LDL with either plasmin, kallikrein, or thrombin, there was no significant (less than 1% of the total radioactivity) generation of ^{35}S-labeled, low-molecular-weight degradation products, as determined by precipitation with trichloroacetic acid. A combination of these three enzymes led to formation of only a slightly larger amount (3%) of such degradation products. None of the treatments produced any measurable fusion of LDL. In sharp contrast, trypsin, α-chymotrypsin, and Pronase did cause extensive formation of trichloroacetic acid-soluble degradation products from apoB-100. Moreover, they triggered fusion of LDL, and the degree of fusion was related to the degree of apoB-100 degradation. As shown in Figure 5B, all the proteases fragmented apoB-100. After incubation with plasmin, a combination of plasmin, kallikrein, and thrombin, trypsin, α-chymotrypsin, or Pronase, no intact apoB-100 remained, whereas after incubation with kallikrein or thrombin, some residual intact apoB-100 was still observable. When LDL was incubated either with a mixture of plasmin, kallikrein, and thrombin or with α-chymotrypsin, there was no release of higher-molecular-weight fragments of apo B-100, as determined using size exclusion gel chromatography (data not shown). The results of these experiments show that mere fragmentation of apoB-100 is not sufficient to trigger LDL fusion; for fusion, loss of apoB-100 material is also required.

Figure 6 shows the equilibrium density centrifugation profiles (in sucrose gradients) of native LDL and of LDL proteolyzed in the presence of α-chymotrypsin for various lengths of time. Native LDL formed a nearly symmetrical peak with a mean density of about 1.025 g/mL. After proteolysis for 12 h, the density was lower, the peak having a mean density of about 1.020 g/mL. Moreover, the density of a fraction of the LDL had decreased even more, extending evenly to values of less than 1.010. As proteolytic degradation of LDL proceeded, the size of the 1.020 g/mL peak decreased and progressively more particles shifted to lower densities. Thus, at 72 h of incubation, the majority of particles had densities of less than 1.005 g/mL.

The flotation characteristics of the proteolyzed and fused LDL particles allowed ultracentrifugal isolation of sufficient quantities of particles for cell culture experiments. To investigate whether the fused LDL particles (size distribu-

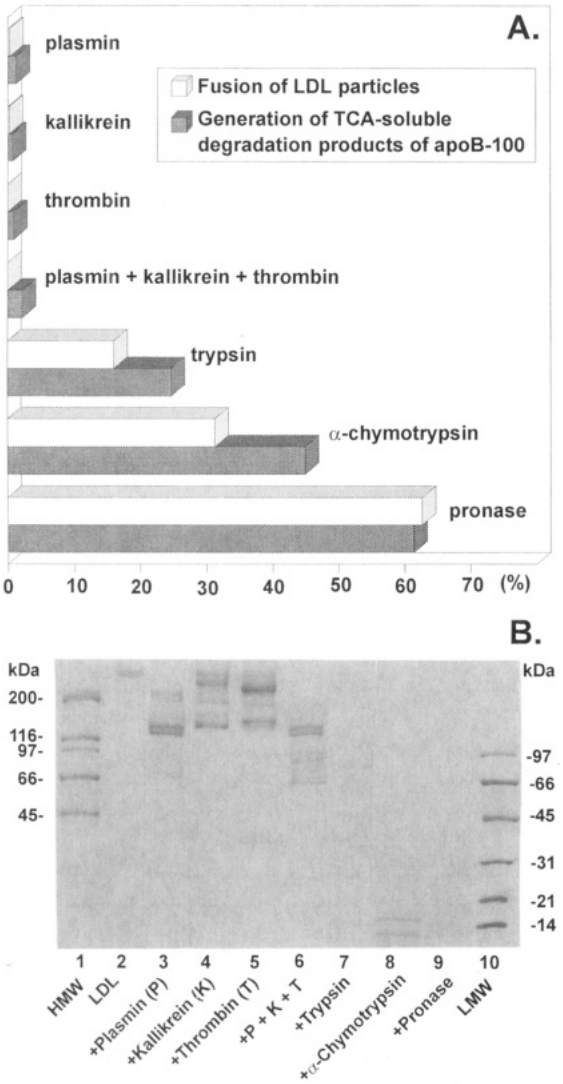


FIGURE 5: (A) Degrees of fusion of LDL and of production of trichloroacetic acid-soluble degradation products of apoB-100 and (B) SDS−PAGE analysis of apoB-100 treatment of LDL with various proteinases. LDL (160 μg) containing both [^{3}H]CE-LDL (2500 dpm/μg of cholesteryl linoleate) and ^{35}S-apoB-100-LDL (900 dpm/μg of protein) was incubated in 160 μL of buffer A at 37 °C for 24 h in the presence of the following proteinases: 6.4 milliunits of plasmin; 8.3 milliunits of kallikrein; 3.3 units of thrombin; a mixture of plasmin (2 milliunits), kallikrein (3 milliunits), and thrombin (1 unit); 32 μg of trypsin; 16 μg of α-chymotrypsin; or 16 μg of Pronase. The control incubation contained no proteases. After incubation, aliquots were taken for Superose gel filtration to determine the degree of LDL fusion (A), for precipitation with trichloroacetic acid to determine the degree of formation of trichloroacetic acid-soluble ^{35}S-apoB-100 degradation products (A), and for 4−20% SDS−PAGE electrophoresis to determine the degree and patterns of apoB-100 fragmentation (B). The degree of fusion of LDL particles and the degree of degradation of apoB-100 of LDL were determined as described in the legend to Figure 3. Electrophoresis was performed as described in Experimental Procedures. Lanes: 1, high-molecular-weight standards; 2, 5 μg of LDL incubated in the absence of proteinases; 3−9, 10-μL samples of proteolyzed LDL corresponding to 10 μg of nonproteolyzed LDL; 10, low-molecular-weight standards.

tion: 63 ± 31 nm; median 58 nm, maximum size 134 nm) are internalized by macrophages, monolayers of mouse peritoneal macrophages were incubated with the proteolyzed and fused LDL particles which had been incubated in the presence of α-chymotrypsin for 72 h and then isolated ultracentrifugally. As an index of LDL uptake, we measured the rate of esterification of [^{14}C]oleate with cholesterol in the macrophages, i.e., the rate of cholesteryl [^{14}C]oleate

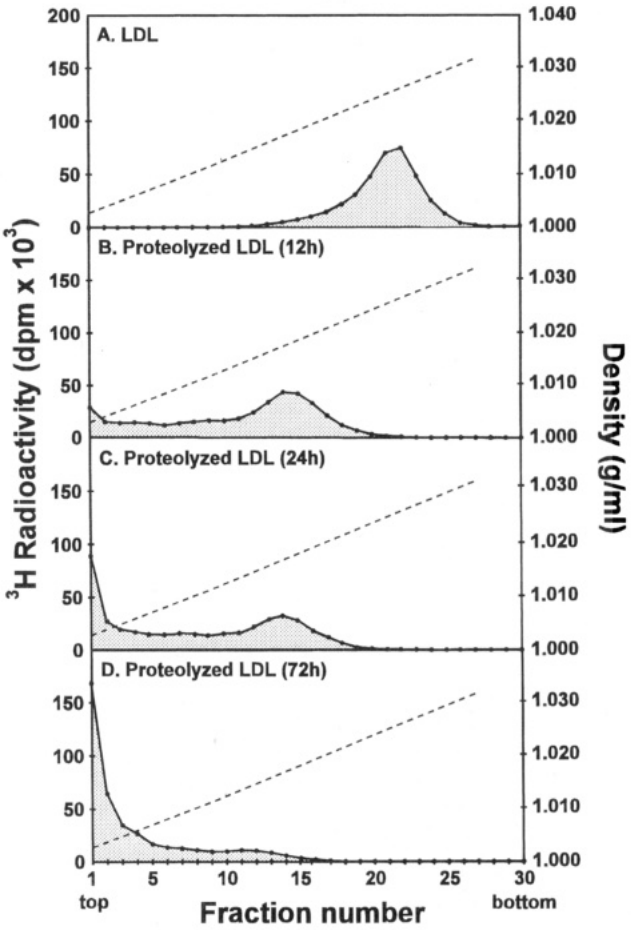
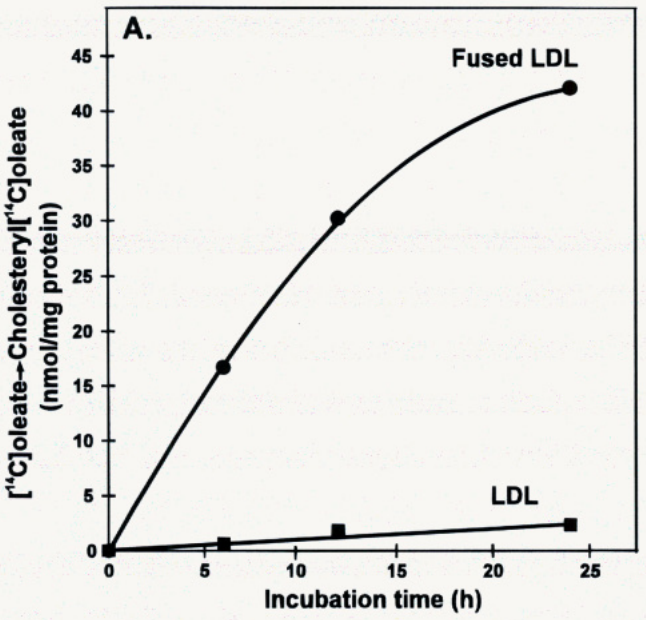
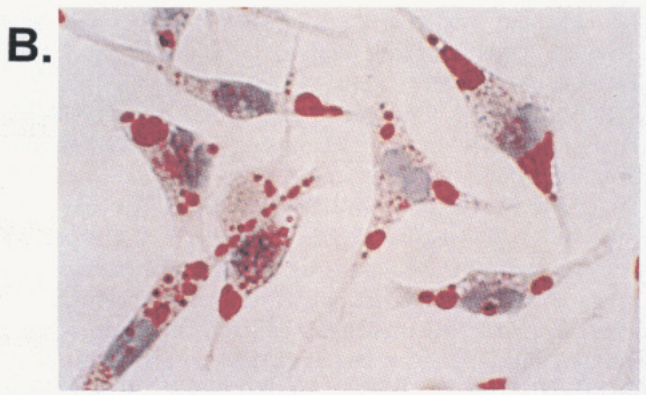


FIGURE 6: Equilibrium density gradient centrifugation of LDL incubated for different periods of time with α-chymotrypsin. [^{3}H]-CE-LDL (200 μg) (2500 dpm/μg) was incubated in 200 μL of buffer A in the absence of α-chymotrypsin at 37 °C for 72 h (A) or in the presence of 20 μg of α-chymotrypsin for 12, 24, and 72 h (B−D). After incubation, the mixtures were layered on top of a linear sucrose gradient and centrifuged at 250000*g* for 72 h. After centrifugation, 400-μL fractions were collected, and their radioactivities were determined. The dashed line indicates the density of sucrose.

synthesis, an intracellular process known to correlate with the amount of cholesterol entering the cell (Goldstein et al., 1979). Figure 7A shows that macrophages incubated with LDL formed only small amounts of cholesteryl [^{14}C]oleate. However, addition of fused LDL particles to the incubation medium led to marked stimulation of cholesteryl [^{14}C]oleate synthesis in macrophages. At 24 h, the amount of cholesteryl [^{14}C]oleate in macrophages incubated in medium containing fused LDL particles was 18-fold that in macrophages incubated in medium containing nonproteolyzed LDL. Oil Red O staining of such macrophages revealed that they contained numerous cytoplasmic lipid droplets typical of foam cells (Figure 7B).

Finally, we compared the rates of macrophage uptake of LDL particles that had been treated with plasmin, kallikrein, or thrombin or their combination or with α-chymotrypsin (Table 1). The variously treated LDL particles were then isolated with a linear sucrose gradient (see Experimental Procedures). The LDL particles treated with plasmin, kallikrein, or thrombin, or with their mixture, had densities similar to that of the control LDL, whereas the α-chymotrypsin-treated particles floated to the top of the gradient (d = 1.005 g/mL). Addition of the proteolyzed but unfused LDL particles (treated with plasmin, kallikrein, or thrombin





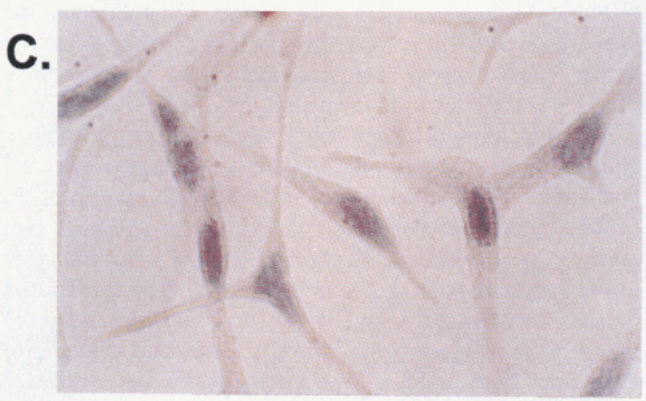


FIGURE 7: Cholesteryl ester formation in macrophages incubated with fused LDL. (A) LDL (8 mg) was incubated in the presence of 800 μg of α-chymotrypsin in 1.5 mL of buffer A at 37 °C for 72 h. After incubation, the fused LDL particles were recovered by ultracentrifugation as described in Experimental Procedures and added to monolayers of mouse peritoneal macrophages. Each dish contained 500 μL of culture medium with 200 μM [^{14}C]oleate–albumin (9400 dpm/nmol) and an amount of either control or fused LDL corresponding to 125 μg of cholesteryl linoleate. After incubation at 37 °C for the indicated times, the cellular content of cholesteryl [^{14}C]oleate in each dish was determined, as described in Experimental Procedures. (B and C) Separate sets of dishes were incubated as above with either fused LDL (B) or control LDL (C). After incubation, the macrophage monolayers were stained with Oil Red O and counterstained with Harris hematoxylin, as described in Experimental Procedures.

or their combination) to the macrophage monolayers did not increase the content of cholesteryl oleate in the cells above the level observed with untreated native LDL. In contrast, the proteolyzed fused particles (treated with α-chymotrypsin) markedly (about 10-fold) increased the cellular content of cholesteryl esters during the incubation for 24 h, showing that proteolytic degradation of LDL renders the particles atherogenic, only if it is accompanied by particle fusion.

Table 1: Stimulation of Cholesteryl Oleate Formation in Mouse Peritoneal Macrophages Incubated with Protease-Treated LDL[a]

treatment	loss of apoB-100 fragments	fusion	particle size [nm (diameter)]	cellular content of cholesteryl oleate (μg/mg of cell protein)
none	(−)	(−)	22 ± 2[b]	11.6 ± 0.5[c]
plasmin (P)	(−)	(−)	23 ± 3	13.1 ± 2.3
kallikrein (K)	(−)	(−)	22 ± 2	8.0 ± 0.7
thrombin (T)	(−)	(−)	23 ± 2	11.0 ± 1.9
P + K + T	(−)	(−)	24 ± 3	12.3 ± 3.0
α-chymotrypsin	(+)	(+)	41 ± 20	101.5 ± 14.3

[a] LDL (1 mg) was incubated in the presence of 50 milliunits of plasmin, 50 milliunits kallikrein, and 20 units of thrombin or a mixture of plasmin (12 milliunits), kallikrein (18 milliunits), and thrombin (6 units), or 100 μg of α-chymotrypsin, in 1 mL of buffer A at 37 °C for 40 h. After incubation, the LDL particles were recovered by linear sucrose gradient equilibrium density ultracentrifugation and their sizes determined by electron microscopy as described in Experimental Procedures. Pooled and concentrated LDL fractions corresponding to 75 μg of cholesteryl linoleate were added to monolayers of mouse peritoneal macrophages containing 600 μL of culture medium and 200 μM unlabeled oleate–albumin. After incubation at 37 °C for 24 h, the cellular content of cholesteryl oleate in each monolayer was determined by HPLC, as described in Experimental Procedures. [b] Median ± SD (n = 250). [c] Mean ± SD (n = 3).

DISCUSSION

This study shows that proteolytic degradation of the apoB-100 component of LDL renders the particles unstable and triggers their fusion. The observation provides proteolytic enzymes with a novel function in the extracellular processing of LDL and also points to the critical role that apoB-100 plays in maintaining the integrity of the LDL particles.

The study also revealed that mere fragmentation of apoB-100 is not sufficient to trigger LDL fusion. Thus, proteolytic enzymes such as plasmin, kallikrein, and thrombin, alone or in combination, which each cleave apoB-100 into specific fragments that remain bound to the particles, do not induce fusion. However, another set of proteolytic enzymes, α-chymotrypsin, trypsin, and Pronase, which cleave apoB-100 into fragments that are released from the particles, do trigger particle fusion. We therefore infer that the critical event in proteolytic destabilization of LDL particles is loss of apoB-100 fragments. Moreover, the results suggest that no specific apoB-100 sequences specifically protect LDL particles from fusion. This conclusion is based on the findings that (i) fusion could be induced by proteolytic degradation of LDL with proteases having different cleavage specificities and that (ii) the rate of fusion was proportional to the extent to which apoB-100 fragments were released by the action of enzymes with a broad specificity. The above observations also suggest that proteolytic destabilization and fusion of LDL were not caused by generation of specific fusogenic fragments of apoB-100. Interestingly, Chao et al. (1992) have observed that pretreatment of LDL with trypsin is necessary for cholesteryl ester hydrolase-induced fusion.

Which proteases could potentially trigger fusion of LDL in the arterial intima, the site of atherogenesis? As LDL

enters the intima, it crosses the endothelial cell layer and on reaching the subendothelial space becomes surrounded by intimal parenchymal cells consisting mostly of smooth muscle cells (Ross, 1986). In addition, three types of blood-borne cells, mast cells (Kaartinen et al., 1994a,b), macrophages (Mitchinson & Ball, 1987), and T lymphocytes (Jonasson et al., 1986), are present in the arterial intima. Our previous studies on the proteolytic fusion of LDL were conducted with the proteolytic enzyme chymase derived from rat serosal mast cells (Kovanen, 1993). Human chymase, like rat chymase, is an endopeptidase with similar (broad) specificity to α-chymotrypsin; it is a component of the cytoplasmic secretory granules of mast cells and is tightly bound to the heparin proteoglycans of human mast cell granules. Therefore, probably the findings with rat mast cell chymase can be extended to include the human arterial intima, since human mast cells were recently found to secrete their proteolytic enzymes (chymase and tryptase) particularly at intimal sites affected by atherosclerotic changes (Kaartinen et al., 1994b). However, the chymase-dependent solid-phase systems, in which both chymase and LDL are bound to proteoglycans, may not be directly comparable to the current system, which, by being a fluid-phase system, provides a model for studies of interactions between unbound proteases and unbound LDL in the intimal fluid.

Macrophages likewise secrete proteolytic enzymes potentially capable of proteolyzing apoB-100 of LDL. Thus, both lysosomal hydrolases and neutral proteinases are secreted upon challenge with appropriate agents, such as immune complexes, complement components, lymphocyte-derived factors, and phagocytic stimuli (Schnyder & Baggiolini, 1978; Davies & Bonney, 1980; Campbell et al. 1989). Moreover, lysosomal enzymes, such as cathepsin B, are released from macrophages when they are incubated with acetylated or malondialdehyde LDL (Hartung et al., 1985), and it has been shown that the lysosomal enzymes released from macrophages proteolyze apoB-100 and enhance uptake of LDL by these cells in vitro (Leake et al., 1990). However, the mechanism by which LDL particles become atherogenic, i.e., the change that leads to their enhanced uptake by macrophages, was not determined in the above studies. The present results tempt us to speculate that particle fusion may have been due to proteolytic degradation of LDL by the various enzymes derived from macrophages and that the increased uptake of LDL by macrophages was at least partly due to fusion of LDL.

The smooth muscle cells which form the bulk of the cellular mass of the intima should also be considered as cells possibly contributing to the extracellular proteolytic modification of LDL in the intima. One example of protease release from smooth muscle cells in the intima is provided by an animal model, the spontaneously hypertensive rats. In these animals, death of smooth muscle cells in the vessel wall is accompanied by release of lysosomes which remain enzymatically active in the extracellular space (Seydewitz & Staubesand, 1988). In addition, the matrix metalloproteinases secreted by migrating smooth muscle cells and by smooth muscle cells participating in tissue remodeling in the atherosclerotic arterial intima could contribute to the generation of LDL-derived lipid droplets in their microenvironments. Finally, the human arterial intima may contain a few cytolytic T cells (Jonasson et al., 1986), and when activated, these also release serine proteases (Kramer & Simon, 1987).

What light do these findings throw on the various stages of the atherosclerotic process itself? The initial morphologic sign of cholesterol accumulation in the arterial intima is the appearance of extracellular lipid droplets (and vesicles) in subendothelial locations. Thus, even in the grossly normal human arterial intima, extracellular lipid droplets can be observed in the subendothelial layer (Pasquinelli et al., 1989). In rabbits also, the earliest sign of local cholesterol accumulation is the subendothelial appearance of lipid droplets. Accordingly, only 2 h after intravenous injection of human LDL, subendothelial lipid droplets appear in the subendothelial space (Nievelstein et al., 1991). Although this experimental design allowed the authors to conclude that the droplets are derived from LDL, they could offer no mechanistic explanation for their generation. The current observations in vitro suggest one potential mechanism for the local generation of such lipid droplets, the fusion of proteolyzed LDL particles. In addition to proteolytic modification of LDL, lipolytic and oxidative modifications of LDL must also be considered as potential mechanisms leading to fusion of the particles. Indeed, experiments in vitro have suggested that, in addition to producing aggregates of LDL particles, lipolytic and oxidative modifications of LDL may also generate larger particles from LDL (Tabas et al., 1993; Dobrian et al., 1993).

Subendothelial foam cells are formed as cytoplasmic cholesteryl ester droplets accumulate in the macrophages. The cholesteryl esters in these foam cells are currently thought to be derived from LDL particles which have been modified in the intimal space. The results of the current work demonstrate that proteolyzed LDL particles, which appear as lipid droplets, are avidly ingested by macrophages, suggesting that the extracellular lipid droplets in the arterial intima could also be a source of the cholesteryl esters in foam cells. This possibility is supported by the recent observations of Pasquinelli et al. (1989), who found that the appearance of foam cells in the subendothelial space of the human carotid intima was accompanied by disappearance of the extracellular lipid droplets.

Recently, more direct evidence has been observed for the ability of the aortic lipid droplets to induce foam cell formation. Thus, Steinbrecher and Lougheed extracted LDL from human atherosclerotic aortic intima and found that uptake of the extracted LDL was accelerated by macrophages in vitro (Steinbrecher & Lougheed, 1992). The accelerated uptake could be attributed largely to scavenger receptor-independent phagocytosis of three types of LDL-containing aggregates (clusters of LDL-sized particles, large vesicular structures, and lipid droplets). In the aggregates, apoB-100 was fragmented, and intrerestingly, the rate of uptake of aortic LDL correlated with the degree of apoB-100 fragmentation. Moreover, the aggregates were taken up by macrophages more efficiently than native-sized LDL particles. Since mild oxidative changes were found in the aortic LDL, the authors suggested that oxidation of the LDL particles in the human aortic intima had caused the fragmentation of apoB-100 and possibly also had contributed to the aggregation of LDL. The current observations on the proteolytic modification of LDL with apoB-fragment release offer an additional possible mechanism for fragmentation of apoB-100 of the aortic LDL particles and for the formation of at least one type of the LDL "aggregates", the lipid droplets, which may then be rapidly ingested by macrophages. The exact way in which mouse peritoneal mac-

rophages recognize the proteolyzed and fused LDL particles remains to be investigated. Since the majority of the fused particles had diameters less than double the diameter of an LDL particle, the fused particles appear to be too small to trigger phagocytosis by macrophages. The possible uptake mechanisms would then include receptor-mediated endocytosis of single fused particles and phagocytosis (receptor-mediated or nonspecific) of multimeric (aggregated) fused particles.

Since the macrophages recognized the fused LDL particles themselves (without any carrier molecules being present), the current mechanism of foam cell formation clearly differs from the previously described proteolytic system, in which proteolyzed LDL particles fused on the surface of mast cell granule remnants, and the fused LDL particles were then passively carried into macrophages as the macrophages ingested the LDL-loaded remnants. Thus, the results corroborate the novel idea that proteolyzed LDL particles may fuse in the arterial intima even while floating free in the intimal fluid. More generally, the present experimental result is a useful addition to our knowledge of the classification of the neutral proteases which may be involved in the proteolytic modification of LDL, with ensuing particle fusion in the human arterial intima.

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