Aggregation and fusion of modified low density lipoprotein

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Abstract In atherogenesis, low density lipoprotein (LDL, diameter 22 nm) accumulates in the extracellular space of the arterial intima in the form of aggregates of lipid droplets (droplet diameter up to 400 nm). Here we studied the effects of various established in vitro LDL modifications on LDL aggregation and fusion. LDL was subjected to vortexing, oxidation by copper ions, proteolysis by α-chymotrypsin, lipolysis by sphingomyelinase, and nonenzymatic glycosylation, and was induced to form adducts with malondialdehyde or complexes with anti-apoB-100 antibodies. To assess the amount of enlarged LDL-derived structures formed (due to aggregation or fusion), we measured the turbidity of solutions containing modified LDL, and quantified the proportion of modified LDL that *1*) sedimented at low-speed centrifugation (14000 *g*), *2*) floated at an increased rate at high-speed centrifugation (rate zonal flotation at 285000 g_{max}), *3*) were excluded in size-exclusion column chromatography (exclusion limit 40 MDa), or *4*) failed to enter into 0.5% FastLane agarose gel during electrophoresis. To detect whether particle fusion had contributed to the formation of the enlarged LDL-derived structures, particle morphology was examined using negative staining and thin-section transmission electron microscopy. We found that *1*) aggregation was induced by the formation of LDL-antibody complexes, malondialdehyde treatment, and glycosylation of LDL; *2*) fusion of LDL was induced by proteolysis of LDL by α-chymotrypsin; and *3*) aggregation and fusion of LDL were induced by vortexing, oxidation by copper ions, and lipolysis by sphingomyelinase of LDL. The various modifications of LDL differed in their ability to induce aggregation and fusion.—**Pentikäinen, M. O., E. M. P. Lehtonen, and P. T. Kovanen.** Aggregation and fusion of modified low density lipoproteins. *J. Lipid Res.* 1996. **37:** 2638–2649.

Supplementary key words LDL • modification • atherosclerosis

In atherosclerosis, lipids initially accumulate in the subendothelial extracellular space of the grossly normal human arterial intima in the form of droplets and vesicles (1, 2). In experimental systems, a similar accumulation of modified lipids can be achieved within hours. Thus, in rabbit aortic intima 2 h after infusion of human low density lipoprotein (LDL) into the systemic circulation (3) or in cardiac valves of the rabbit after 4-h incubation with human LDL in vitro (4), clusters of lipid droplets can be observed with special electron microscopic techniques. These observations strongly suggest that the droplets and vesicles accumulating in the human arterial intima during the initial phases of atherosclerosis are derived from LDL particles.

For plasma LDL (diameter 22 nm) to appear in the intima as extracellular lipid droplets (100–400 nm in diameter), the LDL particles must undergo modification of a type that leads to fusion of the particles. One such modification appears to be proteolytic modification of LDL, which has been studied extensively in vitro using either α-chymotrypsin (5, 6) or the chymotryptic protease chymase of mast cells (7, 8). Other modifications of LDL resulting in the formation of enlarged LDL-derived structures have also been studied (9–15), but their ultrastructural forms, consisting of either lipid droplets or aggregates of native-sized LDL particles attached to each other, have not been studied systematically.

In this study, we used several of the established methods for modifying LDL and focused on differences in the abilities of the different modifications to generate aggregation or fusion of the particles. We found that all of the tested LDL modifications led to aggregation of LDL, but only those that caused extensive changes in the components of the particle surface resulted in particle fusion.

MATERIALS AND METHODS

Materials

[1,2-^{3}H]cholesteryl linoleate and N-succinimidyl[2,3-^{3}H]propionate (^{3}H-Bolton-Hunter-reagent) were from

Abbreviations: anti-apoB-100, antibody to human apolipoprotein (apo)B-100; BSA, bovine serum albumin; CL, cholesteryl linoleate; α-CT, α-chymotrypsin; LDL, low density lipoproteins; MDA, malondialdehyde; PBS, phosphate-buffered saline; SM, sphingomyelin; SMase, sphingomyelinase; TBARS, thiobarbituric acid-reactive substances; TCA, trichloroacetic acid.

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Amersham; FastLane agarose and NuSieve GTG low-melting-point agarose from FMC BioProducts; α-chymotrypsin (from bovine pancreas) and sphingomyelinase (from *Bacillus cereus*) from Sigma; and Superose 6 HR 10/30 columns from Pharmacia LKB Biotechnology. Celite 545 (acid-washed) was from Fluka, Dulbecco's phosphate-buffered saline (PBS) from GIBCO, Glycogel B from Pierce, anti-human apolipoprotein B-100 antibody (anti-apoB-100) from Boehringer Mannheim, copper(II) sulfate pentahydrate and ethylenedinitrilo tetraacetic acid disodium salt dihydrate (EDTA) from Merck, malonaldehyde bis(dimethyl acetal) from Aldrich, and D(+)-glucose from BDH Laboratory Supplies. Cholesteryl ester transfer protein (CETP) was a kind gift from Drs. C. Ehnholm and M. Jauhiainen, National Public Health Institute, Helsinki, Finland.

Preparation and labeling of LDL

Plasma was obtained from healthy volunteers by plasmapheresis at the Finnish Red Cross (Helsinki). The plasma was immediately supplemented with 100 μg/ml gentamycin, 50 μg/ml chloramphenicol, and 3 mM EDTA. Isolation of LDL (d = 1.019–1.050 g/ml) by sequential ultracentrifugation was started within 2 to 3 h after plasmapheresis (16). The isolated LDL was dialyzed extensively against buffer A (150 mM NaCl, 1 mM EDTA, pH 7.4), filtered through 0.22-μm filter, fractionated, and stored at 4°C. The LDL preparations were used within 1 month. The total number of native LDL preparations used for this study was 10. We did not notice differences between different LDL preparations used in this study. The LDL was labeled with [^{3}H]cholesteryl linoleate ([^{3}H]CL-LDL) as described previously (5). Apolipoprotein B-100 (apoB-100) of LDL was tritiated by the Bolton-Hunter procedure (17) to yield [^{3}H]apoB-100. The amounts and concentrations of LDL are expressed in terms of protein. Protein was determined by the procedure of Lowry et al. (18) with bovine serum albumin as standard.

Modifications of LDL

LDL was modified by the following methods.

Vortexing of LDL. LDL (1 mg/ml of protein, corresponding to 1.8 μM) was vortexed in 0.5–2 ml of buffer B (150 mM NaCl, 10 mM Tris-HCl, 1 mM EDTA, pH 7.4) at room temperature for 60 sec, or the period of time indicated, in a conical 10-ml tube with a table vortex (Scientific Industries' Vortex-Genie 2) at full speed.

Generation of LDL-antibody complexes. LDL (1 mg/ml) was treated with anti-human apoB-100 antibody by adding 50 μl or indicated amounts of anti-apoB-100 to 250 μl LDL in buffer B.

Proteolytic treatment of LDL. LDL (1 mg/ml) was proteolyzed with α-chymotrypsin (0.1 mg/ml) in buffer B at +37°C for 72 h, or the period of time indicated. The degree of proteolysis was determined by measuring the amount of trichloroacetic acid (TCA)-soluble [^{3}H] apoB-100 radioactivity.

Oxidation of LDL. LDL (1 mg/ml) was oxidized by incubation in PBS supplemented with 5 μM copper sulfate at +37°C for 72 h, or the period of time indicated. The degree of LDL oxidation was determined by measuring the amount of thiobarbituric acid-reactive substances (TBARS) formed essentially as described by Hessler et al. (19), the electrophoretic mobility of oxidized LDL on cellulose acetate, and the amount of TCA-soluble [^{3}H]apoB-100 radioactivity.

Lipolysis of LDL. LDL (1 mg/ml) was lipolyzed with sphingomyelinase (10 U/l) in PBS supplemented with 0.5 mg/ml BSA at +37°C for 24 h, or the period of time indicated. The amount of sphingomyelin degraded was measured by thin-layer chromatography (12).

Treatment of LDL with malondialdehyde (MDA). LDL was modified with MDA by incubating LDL (final concentration 1 mg/ml) with increasing amounts of MDA in an ice bath for 3 h. After incubation, the LDL was extensively dialyzed against buffer A. The extent of modification was determined by measuring the amount of TBARS in the LDL.

Glycosylation of LDL. LDL (1 mg/ml) was glycosylated by incubating it in buffer A supplemented with 200 mM glucose for 7 days, or the period of time indicated. The extent of LDL glycosylation was determined with boronate affinity chromatography (20), and by measuring the electrophoretic mobility of glycosylated LDL on cellulose acetate.

Analysis of apoB-100 on sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE)

Native and modified LDL (10 μg) were run on Bio-Rad 4–20% SDS-PAGE gels using the Laemmli buffer system under reducing conditions (21). The gels were stained with 0.1% Coomassie Brilliant Blue, destained with 40% (v/v) methanol–10% (v/v) acetic acid, and photographed.

Determination of the amount of enlarged LDL-derived structures

Turbidity of solutions containing modified LDL. The turbidity (absorbance) of modified LDL (250 μl) was measured at 680 nm, using a quartz cuvette with a 1-cm light path with a Biochrom 4060 spectrophotometer (Pharmacia).

Low-speed centrifugation. Modified [^{3}H]CL-LDL (150 μl) was centrifuged at 14000 *g* for 10 min to sediment large aggregates. After centrifugation, the supernatant and pellet were collected for determination of their radioactivities.

Size-exclusion chromatography. Modified LDL (150 μl) was first centrifuged at 14000 *g* for 10 min to sediment

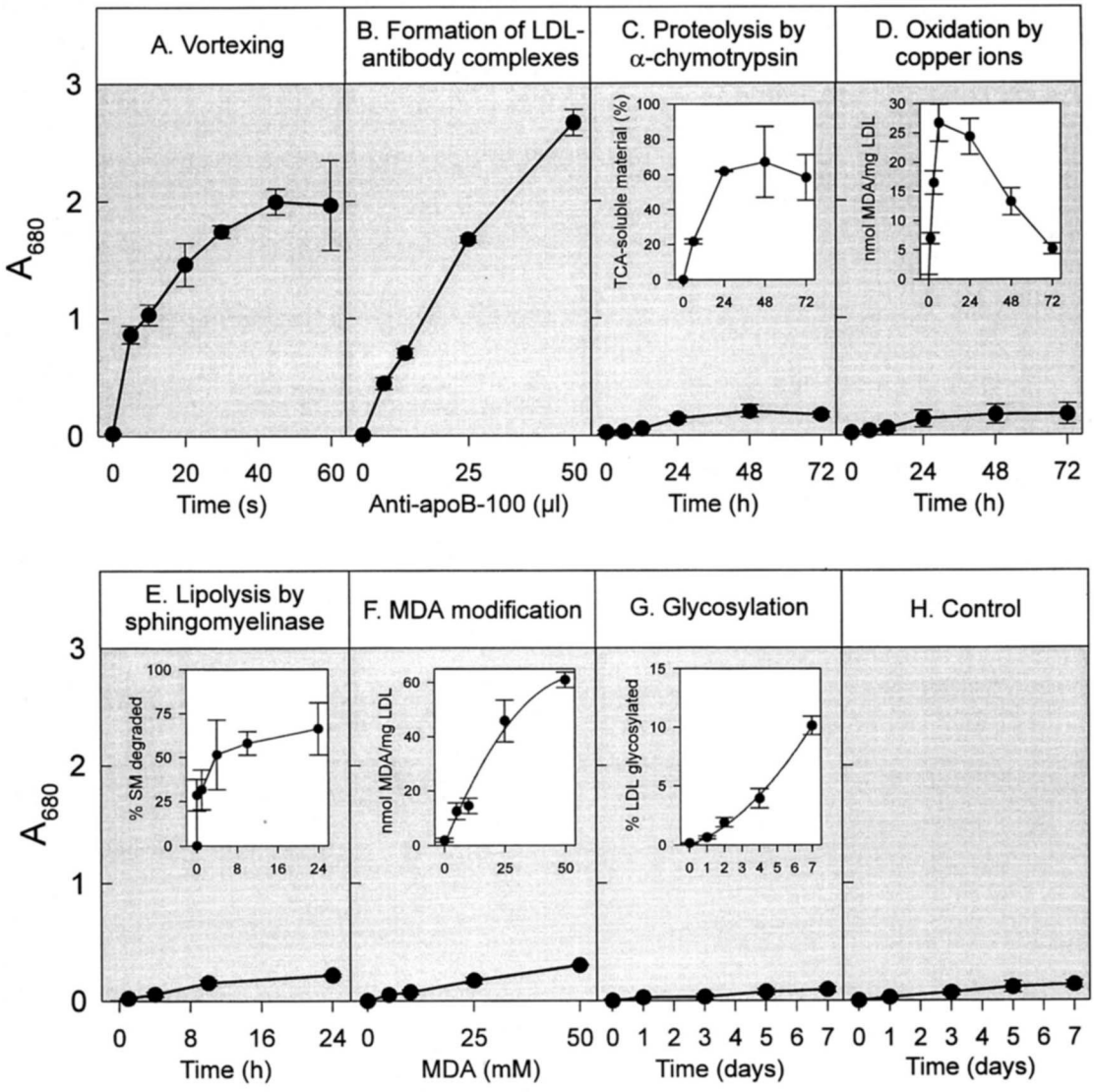

Fig. 1. Turbidity, degree of modification, and SDS-PAGE analysis of variously modified LDL preparations. Panels A to H show turbidities of modified LDL (1 mg/ml) at A_{680}, measured at the indicated time points or at the indicated concentrations. LDL was modified by a variety of methods, and the degree of modification was monitored as described in Materials and Methods. Insets show the degrees of the various modifications. Data points are means of three LDL preparations ± SD.

large aggregates. The supernatant was then applied to a gel filtration system consisting of two Superose 6 HR 10/30 columns connected in series, eluted with buffer B at a flow rate of 0.5 ml/min at 4°C, and 1-ml fractions were collected for analysis of their radioactivities.

FastLane agarose electrophoresis. Modified [^{3}H]CL-LDL (10 μl) was mixed with 10 μl Sudan black, and electrophoresed on a 0.5% FastLane agarose gel in 50 mM barbital buffer, pH 8.8, for 2 h. After electrophoresis, the gel between the application point and the distal end of the visible band was removed from each lane, and melted with a 5-fold excess volume of PBS, and aliquots were taken for measurement of their radioactivities.

Rate zonal flotation of LDL. Centrifugation was performed essentially as described by Polacek, Byrne, and Scanu (22). Briefly, NaBr was added to 500 μl of modified [^{3}H]CL-LDL to yield 40% NaBr (w/vol), and 13 ml of a linear 7.5–30% NaBr gradient (d 1.06–1.29 g/ml) was layered on top of each sample, and centrifuged at 40000 rpm in a SW 40 Ti rotor (Beckman) (285000 g_{max}) at 20°C for 1 h. The gradients were then fractionated into 500-μl aliquots, the radioactivities of which were determined.

Equilibrium density gradient ultracentrifugation of modified LDL. Centrifugation was performed using a discontinuous gradient constructed by layering (from bottom to top) 4 ml of 1.21 g/ml KBr solution containing 250 μg modified [^{3}H]CL-LDL, 3 ml of 1.063 g/ml KBr solu-

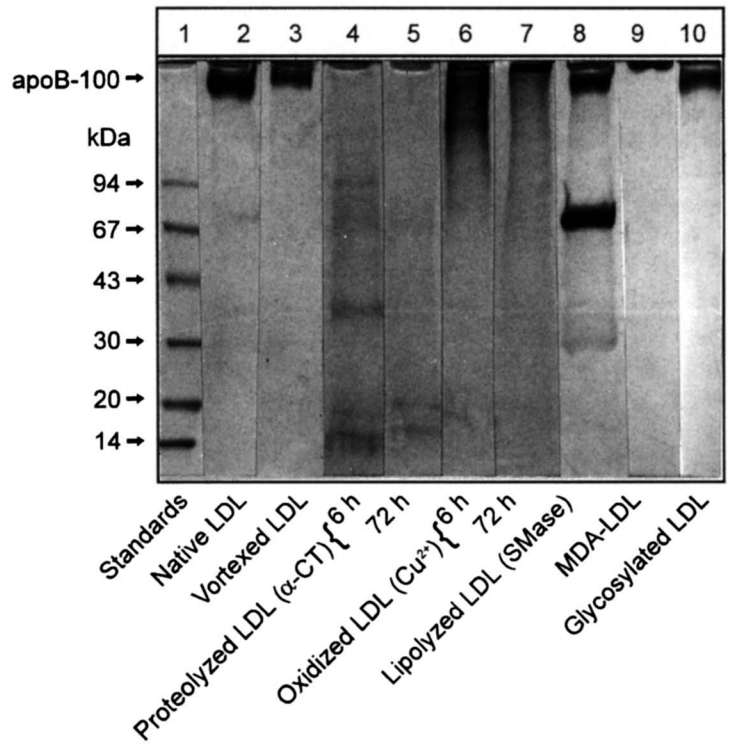

Fig. 1. Panel I: shows SDS-PAGE analysis of apoB-100 of native and of variously modified LDL preparations; α-CT, α-chymotrypsin; MDA, malondialdehyde; SMase, sphingomyelinase.

tion, 3 ml of 1.019 g/ml KBr solution, and 2.5 ml of PBS. Each solution contained 1 mM EDTA. The tubes were centrifuged at 40000 rpm in a SW 40 Ti rotor (285000 g_{max}) at 4°C for 18 h to produce continuous gradients as described (23). After centrifugation, the contents of the tubes were fractionated into 500-μl aliquots and the radioactivities of the aliquots were determined. The densities of the fractions were determined with a refractometer (Bellingham & Stanley).

Determination of the degree of LDL fusion

Particle size was determined with the aid of electron microscopy. For thin-section transmission electron microscopy, 4 mg of modified LDL was concentrated using equilibrium density ultracentrifugations as described above. Concentrated modified LDL (500 μl) was cast into 2% GTG low-melting point agarose gel. Small pieces of gel were fixed in 3% glutaraldehyde at +4°C for 18 h. Fixed samples were stained with the osmium–tannic acid–paraphenylenediamine technique as described (24) and processed for electron microscopy. For negative staining electron microscopy, the samples were stained with 1% potassium phosphotungstate, pH 7.4. The thin sections and the negatively stained samples were viewed and photographed in a JEOL JEM-1200EX and a JEOL JEM-100CX transmission electron microscope, respectively, at the Institute of Biotechnology, Electron Microscopy, University of Helsinki, Helsinki, Finland.

Statistics

In each experiment, three modified LDL preparations were assayed. The results in each figure are shown as means ± SD (n = 3).

RESULTS

LDL was modified by seven methods, as described in detail in Materials and Methods. To study the effect of the LDL modifications on formation of enlarged LDL-derived structures, the turbidity of the solutions containing modified LDL was measured (**Fig. 1;** panels A–H). Turbidity measurements have been found suitable for quantification of large aggregates (9,25,26). As shown in panel A, vortexing caused a rapid increase in turbidity. Addition of increasing amounts of anti-human apoB-100 antibody also strongly increased the turbidity of the solution (B). The increase in turbidity was almost linear, reflecting formation of LDL-antibody complexes. In contrast to the two above treatments, proteolytic modification of LDL by α-chymotrypsin (C), even though extensive (58 ± 13% of apoB-100 was de-

graded into TCA-soluble products at 72 h; inset), produced only a minor increase in turbidity (C). Similarly, there was only a minor increase in turbidity when LDL was extensively oxidized by copper ions (D). Under the conditions used (incubation in PBS supplemented with 5 μM $CuSO_4$), a rapid increase in TBARS was observed during the first 6 h (maximally 27 ± 3.5 nmol MDA/mg LDL protein; inset), which was then followed by a slower decrease in TBARS. Oxidation also increased the electrophoretic mobility of LDL to 2.2 ± 0.1 relative to native LDL at 72 h, and was accompanied by a small amount of protein degradation (at 72 h, 3.7 ± 0.2% of the apoB-100 was TCA-soluble) (not shown). Modification of LDL by sphingomyelinase also increased the turbidity of the sample only slightly (E) despite the extensive lipolysis of LDL (at 10 h, 58 ± 7% of the sphingomyelin in LDL was degraded; inset). Moreover, modification of LDL with increasing amounts of malondialdehyde produced only a slight linear increase in sample turbidity (F), which was accompanied by a progressive increase in TBARS (61 ± 3 nmol MDA/mg LDL protein at the highest concentration of 50 mM; inset) and in electrophoretic mobility (1.9 ± 0.03 relative to native LDL at the highest concentration of 50 mM). Finally, incubation of LDL with 200 mM glucose for 7 days resulted in glycosylation of 10 ± 0.8% of LDL, as determined by binding of LDL to a boronate affinity column (G; inset). However, glycosylation produced only a minimal increase in sample turbidity (G). After 7 days of glycosylation, LDL had an electrophoretic mobility of 1.2 ± 0.03 relative to native LDL. As a control, LDL was incubated at 37°C for 7 days in the absence of any added modifying compounds. As seen in panel H, only a minimal increase in turbidity was noticed. In subsequent assays LDL was vortexed for 60 sec, 50 μl of anti-apoB-100 was added to 250 μl of modified LDL, LDL was proteolyzed with α-chymotrypsin and oxidized by copper ions for 72 h, and lipolyzed with sphingomyelinase for 24 h. LDL was modified with 50 mM MDA, and glycosylated for 7 days.

Panel I (**Fig. 1**) shows SDS-PAGE electrophoresis of the variously modified LDL preparations. From the gel it appears that apoB-100 remained intact in vortexed (lane 3), lipolyzed (lane 8), and glycosylated LDL (lane 10). ApoB-100 in MDA-LDL did not enter the SDS-PAGE gel, probably reflecting cross-linking of apoB-100. Of the modifications, only proteolysis (lanes 4 and 5) and oxidation (lanes 6 and 7) caused fragmentation of apoB-100. When LDL was proteolyzed with α-chymotrypsin for 6 h, distinct bands extending from 10 to 100 kDa appeared. At 72 h, only two low-molecular-weight bands remained visible (also present in the commercial α-chymotrypsin preparation). In contrast, oxidation of LDL for 6 h generated diffuse bands in the high-molecular-weight range, which upon more extensive modification disappeared without producing low-molecular-weight bands. The major band smaller than apoB-100 in lane 8 is BSA. BSA was added to the incubation buffer when LDL was treated with sphingomyelinase (see Materials and Methods). In the LDL-antibody complexes, the proteins (10–100 kDa) in the polyclonal anti-apoB-100 immunoglobulin preparation dominated and precluded analysis of apoB-100 (not shown).

Another method for detection of large aggregates is low-speed centrifugation. As shown in **Fig. 2,** low-speed centrifugation (14000 *g*) sedimented only vortexed LDL and LDL-antibody complexes. Thus, consistent with the results obtained by measuring the turbidity of the sample, only vortexing and addition of LDL antibody led to extensive aggregation of LDL.

To detect small increases in particle size, i.e., formation of small aggregates of LDL and lipid droplets, we analyzed the variously modified LDL preparations that were shown by the two above methods not to contain large aggregates, on size-exclusion chromatography (exclusion limit 40 MDa), a sensitive method for determining the hydrodynamic radius of particles. The elution profile of native LDL is shown in panel A of **Fig. 3.** Proteolysis by α-chymotrypsin, oxidation by copper ions, and lipolysis by sphingomyelinase (panels B–D), three modifications that did not produce significant increases in turbidity of LDL solutions, had produced particles that eluted in the void volume of the column, i.e., had generated particles of increased size due either to aggregation or to fusion of LDL. In contrast, glycosylation (E), which also had failed to increase the turbidity of LDL solutions significantly, had only a minimal effect on the elution profile of LDL, as compared with that of native LDL.

To quantify the total amount of enlarged particles in the modified LDL preparations, i.e., small and large aggregates, and fused particles, we measured the ability of the various modifications to prevent LDL from entering 0.5% agarose gel. For this purpose, LDL was subjected to different types of modification as above and applied to wells of a 0.5% agarose gel. After electrophoresis for 2 h, the amount of modified LDL in the gel was compared with the amount of native LDL in the gel. As shown in **Fig. 4,** most modifications prevented a fraction of the LDL particles from entering the gel, only malondialdehyde-modified LDL and glycosylated LDL being able to enter the gel to almost the same extent as native LDL (which was taken as 100%).

An increase in particle size through either aggregation or fusion of LDL should increase not only the sedimentation of the particles (above), but also their flotation velocity in a centrifugal field (27). To study the flotation characteristics of the modified LDL particles

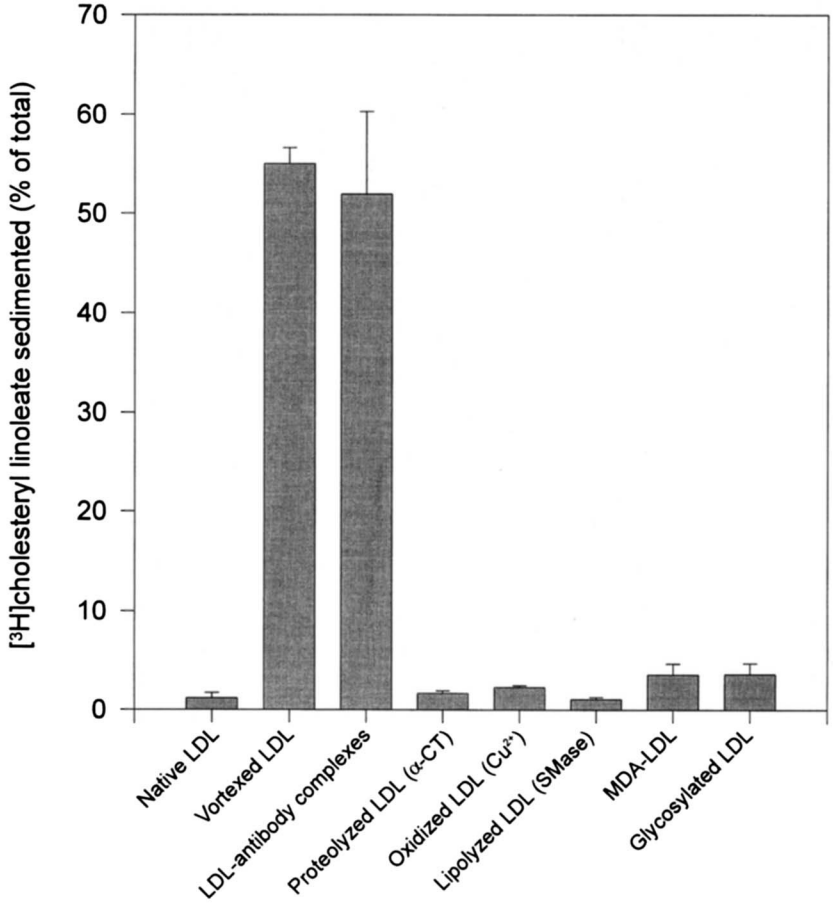

Fig. 2. Low-speed centrifugation of variously modified LDL preparations. LDL was modified by different methods as described in Material and Methods. After each modification, 150 μl of modified [^{3}H]CL-LDL (1 mg/ml) was centrifuged at 14000 *g* for 10 min to sediment large aggregates, and the supernatant and the pellet were collected for determination of radioactivities. The results are expressed as the percentages of [^{3}H]CL radioactivity sedimented. Data points are means of three LDL preparations ± SD. α-CT, α-chymotrypsin; SMase, sphingomyelinase.

in the various preparations, we measured their rates of flotation in a linear 7.5–30% NaBr gradient (1 h at 40000 rpm) in which native LDL was found to float at almost constant speed. Moreover, such a steep gradient and short centrifugation time in a strong centrifugal field minimize the effect of particle density. **Figure 5** shows the flotation profiles of native LDL, vortexed LDL, LDL-antibody complexes, proteolyzed LDL, oxidized LDL, lipolyzed LDL, MDA-LDL, and glycosylated LDL (panels A–H). Vortexing of LDL, formation of LDL-antibody complexes, and proteolysis by α-chymotrypsin and lipolysis by sphingomyelinase of LDL (B, C, D and F, respectively) produced a fast-floating band in addition to the fraction floating at the speed of native LDL. Oxidative modification of LDL by copper ions (E) also formed two fractions, one floating faster and the other slower than native LDL. The majority of MDA-modified LDL (G) floated at the speed of native LDL, but the skewed shape of the peak suggested the presence of a heterogeneous population of faster floating particles. Glycosylation of LDL (H), on the other hand, had no effect on the flotation rate of the LDL particles.

To study the densities of modified LDL, we applied the variously modified LDL preparations to equilibrium density gradient ultracentrifugation. **Figure 6** shows the density profiles of the modified LDL in the preparations (panels A–H). As seen in panel A, native LDL has a peak at the density ~1.04 g/ml, the densities of the particles ranging from 1.03 to 1.05 g/ml. Vortexed LDL (B) produced a narrow turbid band in the density range of native LDL. Addition of anti-apoB-100 antibody increased the density of LDL, thereby reflecting binding of protein to lipoprotein (C). Proteolysis of LDL by α-chymotrypsin produced an array of light particles, their densities ranging from <1.00 to about 1.05 g/ml (D). In contrast, oxidation of LDL by copper ions (E) significantly increased the density of the LDL particles, their densities ranging from 1.06 to 1.09 g/ml. Lipolysis

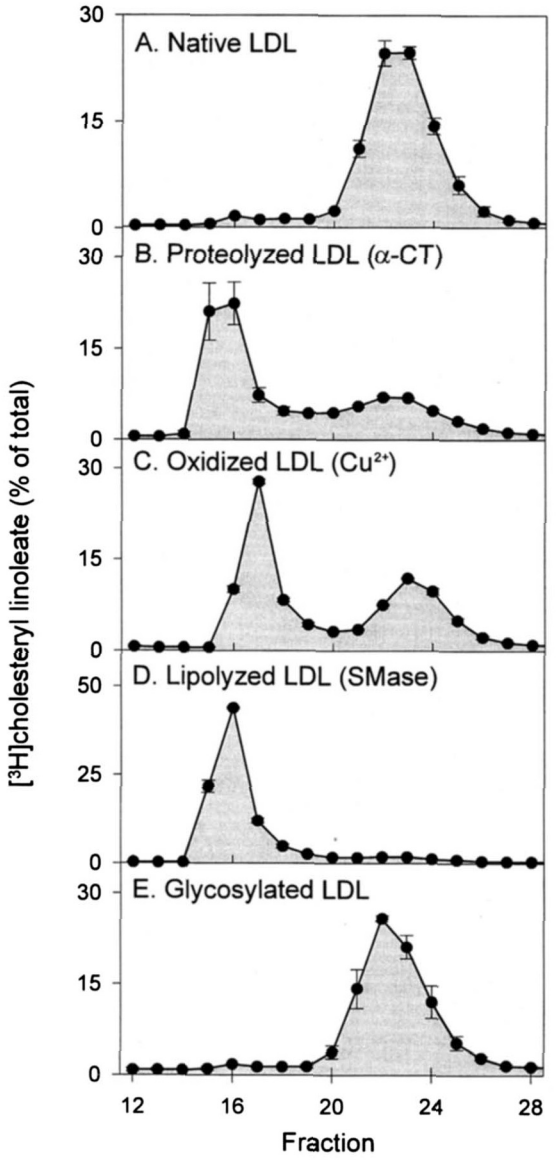

Fig. 3. Gel filtration of variously modified LDL preparations. LDL was modified by different methods as described in Materials and Methods. After each modification, 150 μl of modified LDL (1 mg/ml) was centrifuged at 14000 *g* for 10 min to sediment large aggregates. The supernatant was then applied to a gel filtration system consisting of two Superose 6 HR 10/30 columns connected in series, and eluted with buffer B (150 mM NaCl, 10 mM Tris-HCl, 1 mM EDTA, pH 7.4) at 4°C at a flow rate of 0.5 ml/min. one-ml fractions were collected and analyzed for their radioactivities. Data points are means of three LDL preparations ± SD. α-CT, α-chymotrypsin; SMase, sphingomyelinase.

by sphingomyelinase produced particles in the light end of the density range of native LDL (F). Modification of LDL by MDA (G) increased the density of LDL to some extent, while glycosylation of LDL (H) had no significant effect on LDL density.

Finally, to relate the above physicochemical characteristics of the variously modified LDL particles to their morphology, we prepared samples for both thin-section and negative staining transmission electron microscopy. These methods are complementary in that, in thin-section transmission electron microscopy, it is possible to study the morphology of large particles in aggregates, whereas, in negatively stained preparations, even small increases in size of individual LDL particles are detectable. As aggregates may have formed during sample preparation, no conclusions can be made about the actual degree of aggregation in the samples. **Figure 7** shows thin-section and negative staining (insets) electron micrographs of native LDL (panel A), vortexed LDL (panel B), LDL-antibody complexes (panel C), LDL proteolyzed by α-chymotrypsin (panel D), LDL oxidized by copper ions (panel E), LDL lipolyzed by sphingomyelinase (panel F), MDA-LDL (panel G), and glycosylated LDL (panel H). As shown in panel A, individual native LDL particles can be recognized clearly in the negatively stained sample, and less easily in the thin-section. Native-sized particles were also seen in the LDL-antibody complexes (C), MDA-LDL (G), and glycosylated LDL (H). Enlarged particles were observed after LDL was vortexed (B), proteolyzed (D), oxidized (E), or lipolyzed (F). Vortexing of LDL (B) produced large strand-like structures in which there were *i*) native LDL-like particles, *ii*) large lipid droplets, and *iii*) vesicular structures. Proteolyzed LDL (D) contained lipoprotein-like particles ranging from the size of native LDL to large lipid droplets. Oxidized LDL (E) contained native-sized LDL particles, lipid droplets, and membrane-like structures. Finally, after lipolysis with sphingomyelinase (F), the electron micrographs showed native-sized and small fused LDL particles. In summary, this morphologic study revealed that the various modifications differed in their ability to generate enlarged LDL particles, i.e., to induce fusion of LDL.

DISCUSSION

Of the modifications examined in this study, only proteolysis by α-chymotrypsin, oxidation by copper ions, and lipolysis by sphingomyelinase produced lipid droplets and/or vesicles of similar appearance to those found in the initial atherosclerotic lesions of the arterial intima. The other modifications studied failed to induce fusion of LDL particles, and in the electron microscope the samples showed only native-sized LDL.

What aspects of the modifications of LDL could make the critical differences that lead to either aggregation or fusion of the particles? Aggregation was noticed when anti-apoB-100 was added to LDL, or when LDL was modified chemically by MDA or by nonenzymatic

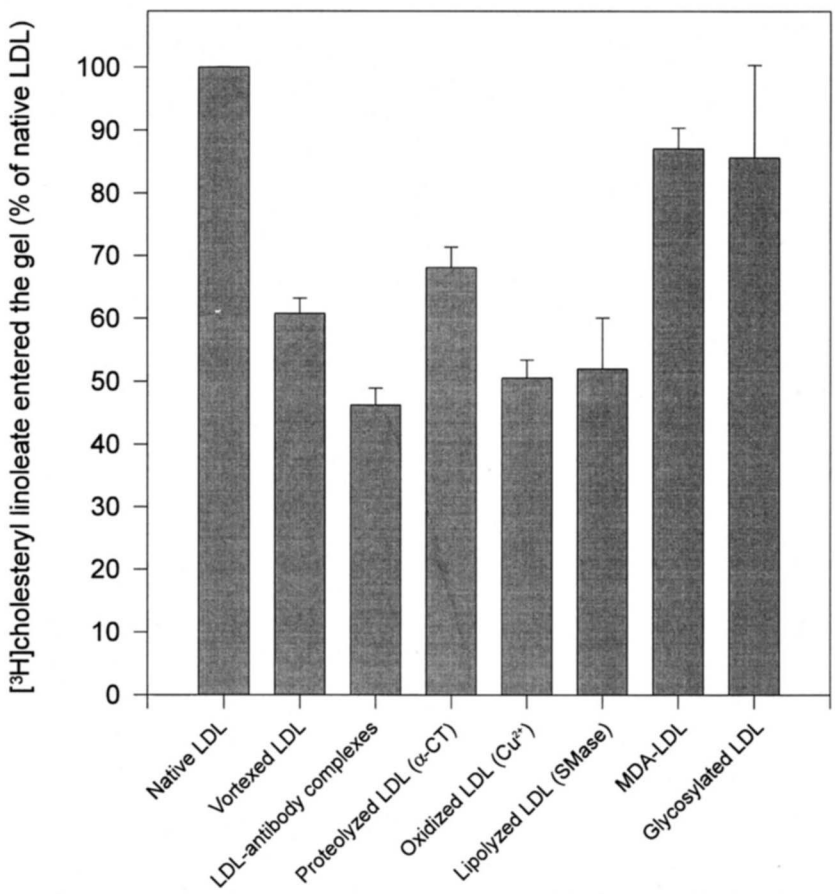

Fig. 4. Entering of variously modified LDL particles into agarose gel. LDL was modified by different methods as described in Materials and Methods. After each modification, 10 μl of modified [^{3}H]CL-LDL (1 mg/ml) was run on a 0.5% FastLane agarose gel for 2 h. The amount of modified LDL entering the gel was measured as described in Materials and Methods, and is expressed in relation to native LDL. Data points are means of three LDL preparations ± SD. α-CT, α-chymotrypsin; SMase, sphingomyelinase.

glycosylation. These modifications all involved addition of exogenous compounds that were able to bind to the apoB-100 component of LDL and so link several LDL particles together. Despite their close proximity, however, the linked particles retained their stability, which allows the formation of aggregates. On the other hand, fusion of LDL was triggered by proteolysis with α-chymotrypsin, oxidation of LDL with copper, lipolysis of LDL with sphingomyelinase, and vortexing of LDL. We have previously shown that fragmentation of the apoB-100 by proteases with narrow substrate specificity did not lead to fusion of LDL, which was triggered only when LDL was proteolyzed by proteases with broad substrate specificities that involved release of apoB-100 fragments (5). Thus, *i*) apoB-100 does not need to be intact for LDL particles to maintain stability, and *ii*) fusion is triggered either by exposure of certain hydrophobic parts of the apoB-100 moiety or by formation of surface defects in the LDL particles upon extensive proteolysis. In addition, fusion is triggered by treatment of LDL with sphingomyelinase, which also produces defects in the surface of the particles. Oxidation of LDL, which leads to changes in both the protein and lipids of LDL, also labilized the LDL particles. However, the results of this study do not enable us to identify the component(s) of oxidized LDL responsible for the observed particle fusion. Oxidized LDL contains MDA adducts, which were shown to aggregate LDL, and which can also promote LDL fusion by bringing labilized particles into close proximity. Finally, the strong mechanical force caused by vortexing of LDL was also able to cause particle fusion. In summary, all the modifications of LDL that triggered particle fusion caused surface defects in LDL.

The purpose of this study was to modify LDL to the morphologic forms that are found in the arterial intima during atherogenesis, and to characterize the particles formed so that comparisons could be made between intimal lipoproteins and in vitro modified LDL. How closely does LDL proteolyzed with α-chymotrypsin resemble the modified LDL found in the arterial intima? In this study, we found that proteolysis of LDL by α-

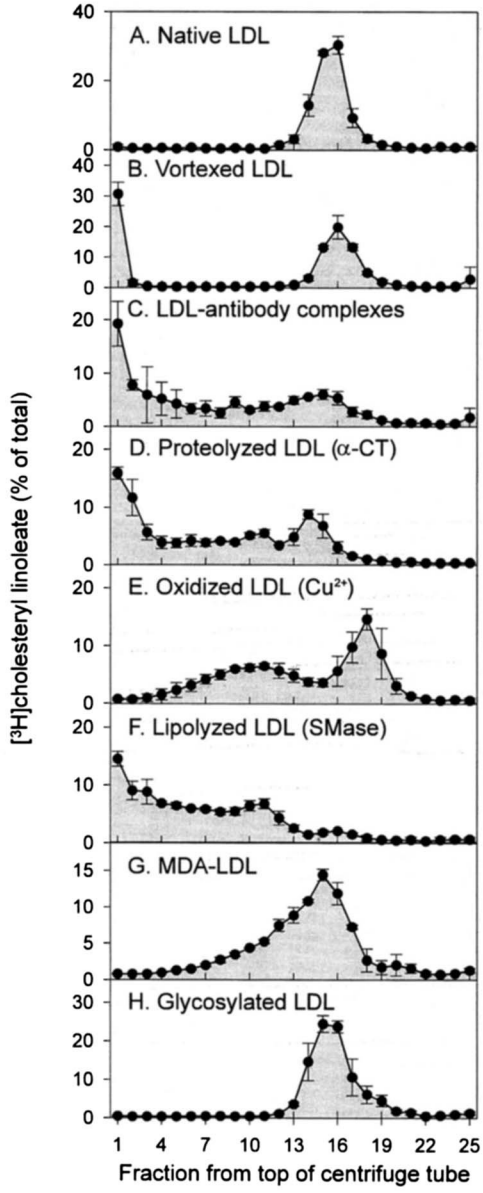

Fig. 5. Rate zonal flotation of variously modified LDL particles. LDL was modified by different methods as described in Materials and Methods. After each modification, 500 μl of modified [^{3}H]CL-LDL (1 mg/ml) was layered under a linear 7.5–30% NaBr gradient (total volume 13 ml), and centrifuged for 1 h at 40000 rpm in a SW 40 Ti rotor (285000 g_{max}) at 20°C. The gradients were then fractionated and the radioactivities of the fractions were determined. Data points are means of three LDL preparations ± SD. α-CT, α-chymotrypsin; SMase, sphingomyelinase.

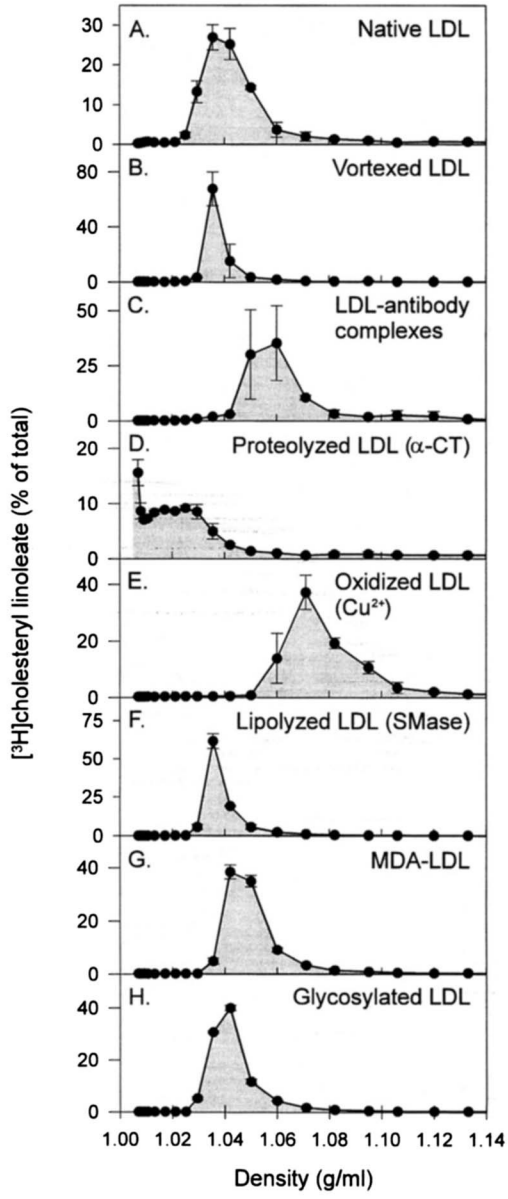

Fig. 6. Density profiles of modified LDL in various preparations. LDL was modified by different methods as described in Materials and Methods. After each modification, 500 μg of modified [^{3}H]CL-LDL was subjected to density gradient ultracentrifugation for 18 h at 40000 rpm in a SW 40 Ti rotor (285000 g_{max}) at 4°C. The gradients were fractionated into 500-μl fractions and the radioactivities of the fractions were determined. Data points are means of three LDL preparations ± SD. α-CT, α-chymotrypsin; SMase, sphingomyelinase.

chymotrypsin caused formation of lipid droplets. The density of the particles was low, and the apoB-100 in the droplets was highly degraded. The morphology of the lipid in the arterial intima (1, 2) resembles that of LDL proteolyzed in vitro. LDL particles isolated from atherosclerotic (28–35), but not from lesion-free (36) arteries have been shown to contain fragmented apoB-100. Particles with densities of less than 1.020 g/ml isolated by Steinbrecher and Lougheed (34) and a fraction of the particles with densities of less than 1.010 g/ml isolated by Chao et al. (37) consisted of lipid droplets with only small amounts of protein, a finding consistent with the hypothesis that these particles had been proteolyzed in vivo. What protease could be responsible for the proteolytic modification of LDL in the arterial intima? A possi-

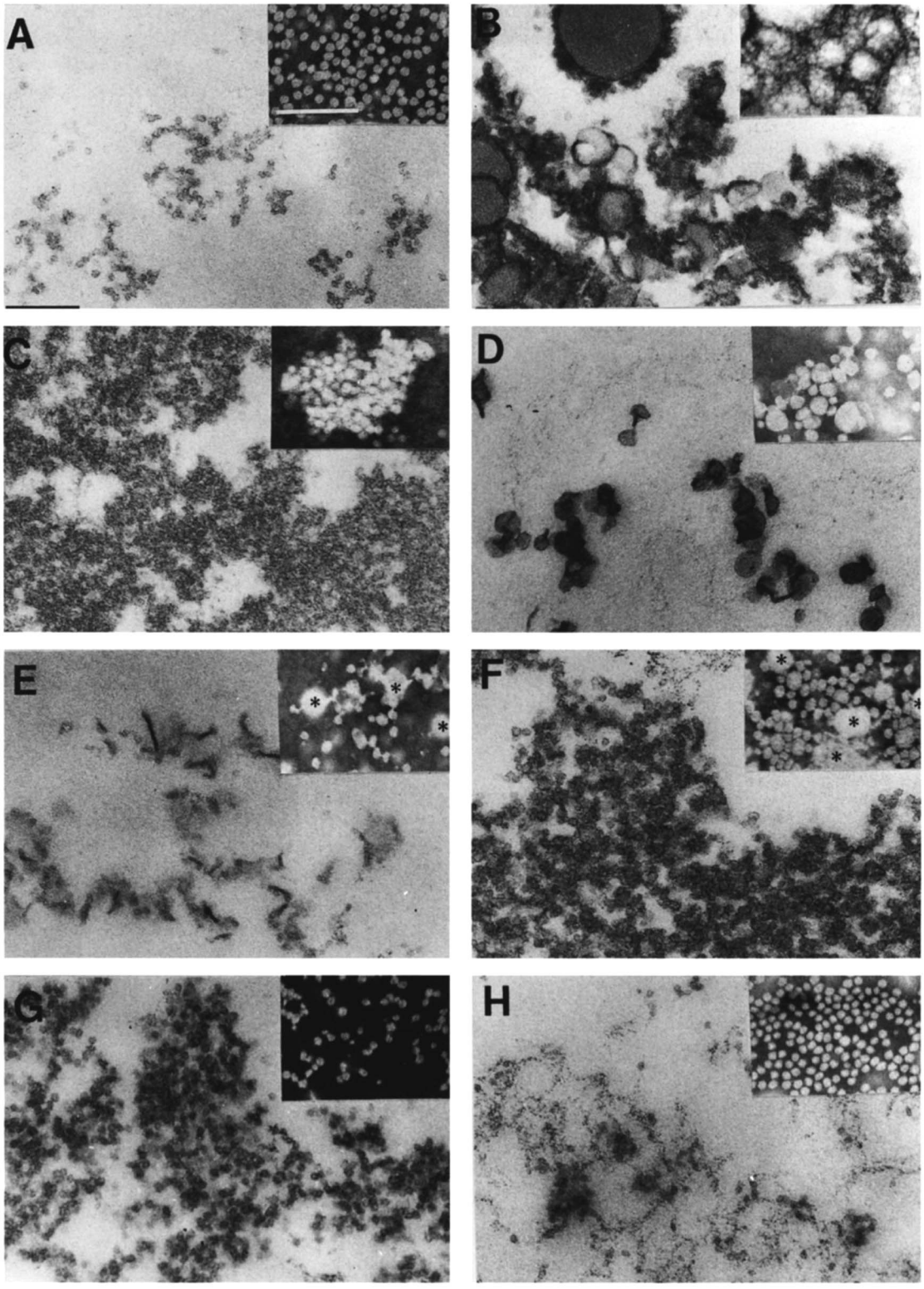

Fig. 7. Electron microscopy of variously modified LDL preparations. LDL was modified by different methods as described in Materials and Methods. Samples were prepared for thin-section and negative staining (insets) electron microscopy, as described in Materials and Methods. Bars in panel A = 200 nm. Original magnifications of thin-section and negative staining electron micrographs were 30000 and 33000, respectively. Panel A, native LDL; panel B, vortexed LDL; panel C, LDL-antibody complexes; panel D, LDL proteolyzed by α-chymotrypsin; panel E, LDL oxidized by copper ions; panel F, LDL lipolyzed by sphingomyelinase; panel G, MDA-LDL; panel H, glycosylated LDL. Asterisks denote structures which were interpreted as artefacts.

ble candidate is mast cell chymase, a chymotryptic enzyme shown to be present in the human arterial intima, especially in areas of lipid accumulation (38).

How does LDL oxidized by copper ions in vitro resemble that present in the arterial intima? The current study and a previous study by Dobrian et al. (14) showed that extensive oxidation of LDL produces particles morphologically resembling those found in the arterial intima. Moreover, fragmentation of apoB-100, increased electrophoretic mobility, and increased density are properties shared by copper-oxidized and arterial LDL (39). There is, in fact, strong evidence that LDL is oxidized to some degree in vivo, although the exact mechanism is not known. Whatever the mechanism, the LDL oxidation in the arterial intima appears to be mild, for aortic LDL has been shown to contain only small amounts of TBARS, to have only a slightly increased density, and to contain only partially degraded apoB-100 (30, 34). Whether it is such mild oxidation in the aortic intima and other types of oxidation (besides copper-mediated oxidation of LDL) that alter LDL morphology to resemble the lipid droplets seen in the subendothelial extracellular space remains to be studied.

How does LDL modified with sphingomyelinase in vitro resemble the modified LDL found in the arterial intima? In keeping with our results in vitro, studies by Xu and Tabas (12) have shown generation of enlarged particles after lipolysis of LDL sphingomyelinase. Furthermore, arteries have been shown to contain sphingomyelinase (40). It has also been shown that aortic sphingomyelinase can act on the LDL retained in aortic strips ex vivo (41). However, the role of sphingomyelinase in generating lipid droplets in the arterial intima is difficult to reconcile with the fact that in esterified cholesterol-rich particles isolated from human atherosclerotic lesions (37) and in LDL isolated from atherosclerotic vascular tissue of WHHL rabbits (31) the relative content of sphingomyelin is increased rather than decreased. This suggests lipolysis of other types of phospholipids, e.g., phosphatidylcholine. Interestingly, atherosclerotic lesions have recently been shown to contain nonpancreatic secretory phospholipase A_2 (42).

Indeed, several of the in vitro modifications of LDL mimic some characteristics of the lipid droplets isolated from the atherosclerotic plaques of the arterial intima. Both proteolysis and oxidation cause fragmentation of apoB-100, and treatment with phospholipase A_2 mimics the oxidative modification of LDL (43). Therefore, it is necessary to analyze the droplets systematically, using several criteria. For example, as shown in this study, the density of proteolyzed LDL was lower and that of oxidized LDL higher than the density of native LDL, features that help to separate these two modifications, both of which generate fragmented apoB-100. In conclusion, as it is likely that the LDL in the arterial intima is modified in multiple ways, careful analysis of the arterial lipid droplets, taking into account morphologic, chemical, and physicochemical characteristics, will be needed in the future.

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