Liposome-like Particles Isolated From Human Atherosclerotic Plaques Are Structurally and Compositionally Similar to Surface Remnants of Triglyceride-Rich Lipoproteins

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Abstract Recent studies have demonstrated the presence of unesterified cholesterol-rich, liposome-like vesicles in the extracellular space of atherosclerotic lesions in humans and animals. Liposome-like vesicles accumulate in the subendothelial space in rabbits within 2 weeks of initiation of cholesterol feeding, well before foam cells appear. These observations suggest that extracellular liposome-like vesicles may play a pivotal role in atherogenesis. The origin of these particles is unknown. We report a combination of in vivo and in vitro experiments that suggest a novel origin for these liposome-like vesicles. We demonstrate that the liposome-like particles isolated from postmortem human atherosclerotic plaques are rich in intact apolipoprotein (apo) A-I, C apolipoproteins, and sphingomyelin. We show that the in vivo derived particles are virtually identical, structurally and compositionally, to liposome-like lipolytic surface remnants of triglyceride (TG)-rich lipoproteins produced during in vitro lipolysis of hypertriglyceridemic serum. In vitro lipolysis of isolated very-low-density lipoprotein has shown that the lipoprotein surface remnants remain attached to the core remnants in the absence of high-density lipoprotein (HDL), dissociate to form liposome-like vesicles in the presence of low levels of HDL, and are assimilated into HDL to form larger HDL particles in the presence of excess HDL. Thus, the in vitro produced, liposome-like particles represent a complex of lipolytic surface remnants of TG-rich lipoproteins and apo A-I derived from HDL. Two possible origins have been suggested for the extracellular liposome-like vesicles in atherosclerotic plaques: (1) modified, aggregated, and/or degraded LDL particles entrapped in an intimal matrix and (2) intracellular lipid products of arterial wall cells. Neither possibility directly explains the presence of A-I and C apolipoproteins and excess sphingomyelin that we observe. We propose as an alternate explanation that the in vivo liposome-like particles are lipolytic surface remnants of TG-rich lipoproteins. We further suggest that these remnants are produced in the intimal space by undefined processes and/or are transected into the intima from the plasma compartment as a product of normal lipolysis gone awry. We conjecture that one role of HDL may be to assimilate the highly atherogenic liposome-like particles in a (1) "mop-up" fashion to remove them from the artery wall and/or (2) preventive fashion in the plasma compartment to prevent their transcytosis into the artery wall. The suggestion that elevated concentrations of surface remnants act as a "sink" for apo A-I can also account for the well-established but poorly understood link between hypertriglyceridemia and low HDL. (Arterioscler Thromb. 1994;14:622-635.)

Key Words • atherosclerotic plaques • triglyceride-rich lipoproteins • lipolytic surface remnants • liposome-like vesicles • HDL • lipolysis

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Cholesterol deposition in the arterial intima is characteristic of human atherosclerotic plaques.1 These deposits represent both intracellular lipid droplets (foam cells) and extracellular lipoprotein (VLDL)- and low-density lipoprotein (LDL)-like particles. The cholesterol deposits in fibrous plaques are predominantly extracellular.2 Several recent studies have shown that atherosclerotic lesions in both humans and experimental animals contain numerous large, extracellular, liposome-like particles, which differ from extracellular spheroidal very-low-density lipoprotein (VLDL)- and low-density lipoprotein (LDL)-like particles and intracellular lipid droplets in foam cells with respect to size and chemical composition.3-10 In animal models these particles accumulate before foam cells appear, emphasizing the primacy of these particles in atherogenesis.5 Frank and Fogelman6 have indicated that these vesicles often appear in clusters in atherosclerotic lesions; many of these clustered vesicles seem to be in the process of fusing together. Chao et al11,12 recently isolated and characterized liposome-like vesicles from atherosclerotic lesions of human and rabbits on an atherogenic diet. The liposome-like vesicles had a flotation density of 1.02 to 1.08 g/mL and were poor in core lipids. Mora et al13 reported that the extracellular liposome-like vesicles in atherosclerotic lesions of hypercholesterolemic rabbits colocalized with immunoreactive B apolipoproteins; however, a recent study by these investigators14 found that purified liposome-like vesicles contain no apolipoprotein (apo) B.

Two possible origins have been suggested for the extracellular liposome-like vesicles observed in atherosclerotic plaques: (1) modified, aggregated, and/or degraded LDL particles entrapped in an intimal matrix3-12 and (2) intracellular lipid products of arterial wall cells.13-15 Chao et al17 have suggested that the liposome-like vesicles and cholesteryl ester-rich, spherical, VLDL-like particles in atherosclerotic plaques may have a
common origin or that one may be the precursor of the other. The appearance of liposome-like vesicles in the subendothelial space in cholesterol-fed rabbits is closely associated with the appearance of β-VLDL in plasma. Recent in vivo studies of the uptake of gold-labeled β-VLDL in rabbits showed that β-VLDL–gold complexes colocalized with extracellular liposomes in the artery wall. In vitro studies have shown that aggregation of LDL induced by vortexing or hydrolysis of cholesteryl ester in the core of LDL by bacterial cholesteryl esterase produced multilamellar liposome-like vesicles. Although these vesicles have been shown to have a morphology and lipid composition similar to the liposome-like vesicles in atherosclerotic lesions, the in vivo significance of this observation remains uncertain.

We have observed previously that in vitro lipolysis of VLDL in the presence of low levels of high-density lipoprotein (HDL) produces liposome-like surface remnants that are similar to those in human atherosclerotic plaques. This observation led to the present study: a detailed comparison of the structure and composition of liposome-like surface remnants derived from in vitro lipolysis of VLDL with the liposome-like particles isolated from human atherosclerotic plaques.

Methods

Materials

Human aortas (5 to 24 hours postmortem) were obtained from the Tissue Procurement Facility of the University of Alabama at Birmingham. Fresh normolipidemic and hypertriglyceridemic (HTG; type IV and V) plasma or sera were obtained from volunteers and from the Alabama Regional Blood Center, Birmingham. A monoclonal antibody against human plasma apo A-I (anti-apo A-I-7) was kindly provided by Dr Linda Curtiss, Scripps Clinic and Research Foundation, La Jolla, Calif. Lipoprotein lipase (LpL) was separated from raw bovine milk and purified by the affinity column chromatographic method using heparin-agarose (Bio-Rad Laboratories) as described by Iverius et al. Sodium dodecyl sulfate (SDS) gradient gel electrophoretic analysis of the purified bovine milk LpL showed a single 55 000-D protein band as its major component and two minor bands of molecular weight 16 000 to 18 000 D; the latter represent proteolytic fragments of 55 000-D LpL. Unstimulated macrophages were harvested from the peritoneal cavities of CD1 white male mice (Charles River Breeding Laboratory) according to the method of Edelson and Cohn and plated in six-well culture dishes. After allowing macrophages to adhere to the culture dishes (4 hours after plating), they were washed three times with buffered saline.

Isolation of Lipoprotein-like Particles From Human Aortic Plaques

The aortic segments were placed immediately in chilled, buffered saline (0.01 mol/L Tris, 0.15 mol/L NaCl, pH 7.4) containing 0.1% EDTA, 20 μmol/L butylated hydroxytoluene, and 1 mmol/L phenylmethysulfonyl fluoride. The aortic tissues were then rinsed five times with buffered saline to remove residual blood. The intima-media layers of the aortic tissues were stripped and minced with scissors to <1 mm² in chilled,
Fig 2. Negative-stain electron microscopy of cholesterol-containing lipoprotein-like particles isolated from human aortic atherosclerotic plaques. Lipoprotein-like particles were isolated by buffer extraction and density gradient ultracentrifugation as described in the text. A, Plasma very-low-density lipoprotein; B, very-low-density lipoprotein density fraction isolated from a fatty streak-like lesion; C, plasma low-density lipoprotein (LDL); and D through F, various subfractions of LDL density particles isolated from aortic tissue having raised plaques (fractions in Fig 1D). Samples in D through F are supernatants of pooled LDL density fraction I (D) and pooled LDL density fraction II (E) and pelleted material of pooled LDL density fraction II (F) of Fig 1D. The arrowhead and * mark aqueous cores of single and multilamellar vesicles, respectively.
buffered saline. The minced aortic sections were shaken overnight (16 hours) at 4°C. The aortic tissue suspension was then subjected to low-speed centrifugation (1000 g x 10 minutes) to separate the supernatant from tissue fragments.

A small portion (0.5 mL) of the supernatant was taken for analysis of the lipoprotein cholesterol profile by the vertical autoprofile method, which involved a short (30-minute) density gradient ultracentrifugal separation of each density class of lipoproteins in a vertical rotor and continuous flow monitoring of cholesterol levels in the effluent from the density gradient tube by the use of a Technicon Autoanalyzer. The density of the remaining supernatant fraction was adjusted to 1.30 g/mL with solid KBr, and a two-step density gradient was formed by layering 3 mL of sample under 8 mL of normal saline. The tubes were then subjected to ultracentrifugation in a swinging-out rotor (Beckman SW 41) at 40,000 rpm for 4 hours. The samples in the density gradient tubes were then fractionated with a gradient fractionator (Accurate Chemical & Scientific Co). After measurement of cholesterol in each gradient fraction by an enzymatic assay, the fractions containing VLDL-, LDL-, and/or LDL-like particles were pooled and dialyzed against buffered saline overnight at 4°C.

The dialyzed VLDL-LDL density fractions then were subjected to low-speed centrifugation (6000 rpm x 30 minutes) in a Sorvall RC-3 centrifuge to pellet the multimolecular liposome-like vesicles. The pellet was washed twice with buffered saline and then subjected to a second density gradient ultracentrifugation step. The turbid band formed in the LDL density region was collected with a Pasteur pipette.

The supernatant of the pooled LDL density fraction was fractionated further by affinity chromatography on a heparin-agarose column. Briefly, the supernatant of LDL density fractions from several aortic tissues was pooled, dialyzed against Tris buffer (0.01 mol/L, pH 7.4) containing 0.05 mol/L NaCl, and loaded onto a heparin-agarose column (40-mL bed volume) at 4°C. The absorbance of the effluent from the density gradient tubes was monitored at 280 nm. The aortic LDL fraction that eluted without binding to the column was collected. When the absorbance of the effluent from the column reached baseline, aortic LDL bound to the column was eluted with Tris buffer containing 0.15 mol/L NaCl and subsequently with Tris buffer containing 1.0 mol/L NaCl. The fractions collected from the heparin-agarose column were concentrated by subjecting the fractions containing LDL-like vesicles to isopycnic ultracentrifugation.

Interaction of Aortic Lipoprotein-like Particles With Mouse Peritoneal Macrophages

To determine the ability of lipoprotein-like particles isolated from human aortic plaques to induce foam cells, 4 mL serum-free culture medium (Medium 199) containing 200 μg (cholesterol) of lipoprotein-like particles, plasma LDL, or acetylated LDL per milliliter of medium was added to culture dishes containing 5 x 10⁴ macrophages. In certain experiments 200 to 400 μg HDL cholesterol was added to culture dishes containing 200 μg of lipoprotein-like particles. The culture dishes were incubated for 18 hours at 37°C in a humidified cell-culture incubator with 5% CO₂. After incubation the culture medium was removed, and each well was washed four times with phosphate-buffered saline (0.05 mol/L phosphate, 0.15 mol/L NaCl, pH 7.4). Cellular lipid was extracted twice with 2 mL hexane/isopropanol (3:2, vol/vol) and once with 2 mL hexane. After evaporation of the solvent, total cholesterol was measured by an enzymatic assay method. Cellular lipids were dissolved in 2 mL of 0.1N NaOH, and 0.25- to 0.5-mL aliquots were assayed by the method of Lowry et al. 27

Preparation of Lipolytic Remnants of Triglyceride (TG)-Rich Lipoproteins

Lipolysis of TG-rich lipoproteins was performed in vitro by (1) adding purified bovine milk LpL to HTG serum or to a lipolysis mixture containing isolated VLDL (40 mg/dL cholesterol), fatty acid–depleted bovine serum albumin (6%), and/or isolated HDL (10 to 80 mg/dL cholesterol) and (2) subsequently incubating the mixture at 37°C for 120 minutes. In certain experiments HDL was added to a lipolysis mixture of VLDL and the mixture incubated at 37°C for 4 hours.

To determine the fate of lipolytic remnant products, a trace amount of ¹²⁵I-labeled VLDL was included in certain lipolysis mixtures. HTG serum or lipolysis mixtures incubated with heat-inactivated LpL served as controls. The control and postlipolysis samples of HTG serum or VLDL were subjected to density gradient ultracentrifugation as described in the text. A, A-very-low-density lipoprotein-like particles isolated from a normolipidemic subject; C, multilamellar liposome-like vesicles isolated from a hypertriglyceridemic subject; D, control VLDL isolated from a normolipidemic subject; E, molecular-weight standard. Apo indicates apoprotein; Alb, albumin.
ing the flocculent remnant band were heavily contaminated with LDL. The flocculent remnant band was separated from LDL by centrifuging the dialyzed samples at 6000 rpm for 30 minutes in a Sorvall RC-1 low-speed centrifuge; the flocculent remnants not bound to LDL were pelleted by this step. The purified flocculent remnants were purified further by washing two times with buffered saline and then by performing another density gradient ultracentrifugation in a swing-out rotor. The purified flocculent remnants located in the middle of the density gradient tubes were collected with a long-stem Pasteur pipette.

In other experiments, excess free fatty acid (FFA) associated with the flocculent remnants was removed by adding fatty acid–depleted bovine serum albumin at a 1:4 molar ratio of albumin to FFA and incubating the mixture for 2 hours at 37°C. The FFA-depleted flocculent remnants were then separated from albumin by density gradient ultracentrifugation.

Characterization of Aortic Lipoprotein-like Particles and Lipolytic Remnants of TG-Rich Lipoproteins

The chemical compositions of extracellular lipoprotein-like particles and lipolytic remnant products of TG-rich lipoproteins were determined by measuring the concentrations of protein, phospholipid, unesterified cholesterol, cholesteryl ester, TG, and FFA of purified samples. Unesterified cholesterol and cholesteryl ester were assayed by the enzymatic method described by Allain et al.26 TG and FFA were assayed by the use of Boehringer Mannheim enzymatic assay kits (No. 348292 and 1082). Protein and phospholipid were assayed by the colorimetric methods of Lowry et al.27 and Stewart,28 respectively. Lipid was extracted from aortic lipoprotein-like particles and plasma lipoproteins, and the phospholipid composition was examined by thin-layer chromatography on silica gel plates.29 The relative ratios of sphingomyelin, lysophosphatidylcholine, and phosphatidylcholine in the plates were determined by densitometric scanning of the thin-layer plates with an auto gel scanner (Helena Laboratories).

The apolipoprotein composition of aortic lipoproteins and/or lipoproteins isolated from prelipolysis and postlipolysis serum were examined by SDS gradient gel electrophoresis.30 Apo A-I in SDS gels was probed immunologically after electrophoretic transfer of apo A-I from the gel to a nitrocellulose membrane (Western blotting). Briefly, duplicate SDS gradient gels containing samples of lipoproteins from aortic tissue or plasma were electrophoresed for 6 hours.30 Then one gel was fixed and stained to visualize the protein bands in the gel, and the apolipoproteins in the other gel were transferred to a nitrocellulose membrane.31 The blotted nitrocellulose membrane was reacted with monoclonal antibody A-I-7 specific for human apo A-I and subsequently with goat anti-mouse immunoglobulin G coupled to alkaline phosphatase.

The morphology of lipoprotein and/or liposome-like particles from aortic extracts and plasma was examined by negative staining with 2% potassium phosphotungstate and examining the grid on a Phillips 400 transmission electron microscope.

Results

Characterization of Lipoprotein-like Particles From Human Plaques

Buffer extracts of atherosclerotic plaques from human aortic tissue were subjected to density gradient ultracentrifugation, and the levels of cholesterol in the effluent from the density gradient tubes were continuously monitored. As shown in Fig 1A and 1B, most of the cholesterol in the buffer extracts was associated with the VLDL and LDL–intermediate-density lipoprotein density region; Fig 1C is a lipoprotein cholesterol profile of plasma from a subject with HTG. The ratio of VLDL to LDL–intermediate-density lipoprotein density material from atherosclerotic tissue extracts (A-VLDL and A-LDL, respectively) from different subjects varied considerably. Fatty streak-like lesions had a greater proportion of A-VLDL (Fig 1A), whereas raised, cholesterol-rich plaques contained proportionately more A-LDL (Fig 1B). Little or no cholesterol was detected in the buffer extract from regions of aorta without lesions (data not shown).

Negative-stain electron microscopy of A-VLDL (Fig 2B) showed spheroidal particles larger than plasma VLDL (Fig 2A); spherical particles containing excess surface material in the form of phospholipid vesicular blebs and tabs were detected in certain microscopic fields (Fig 2B, inserts). Similar particles observed in prelysosomal organelles of rat liver have been identified as remnants of TG-rich lipoproteins.32 Compositional analysis of A-VLDL isolated from aortic tissue containing mild fatty streaks showed a lower TG-to-cholesterol ratio (0.57) than plasma VLDL (4.35), and SDS gradient gel bands corresponding to apo A-I, apo B-100, and C apolipoproteins were seen (Fig 3A). We observed that the VLDL fraction isolated from aortic tissue having advanced lesions contained numerous large, aggregate, lipid particles in addition to VLDL-like particles, and these VLDL-density particles contained numerous unidentified protein bands in addition to identifiable A-I and C apoproteins (data not shown).
When buffer extracts of aortic tissues having advanced atherosclerotic lesions were subjected to preparative single vertical-spin ultracentrifugation, two to three overlapping lipoprotein bands were seen in the LDL density region of the density gradient tube (Fig 1D). Two A-LDL fractions (fractions I and II of Fig 1D) were pooled, dialyzed against buffered saline, and centrifuged at 6000 rpm for 30 minutes. Little or no material was pelleted from fraction I, but approximately 60% of the cholesterol associated with fraction II was pelleted.

Negative-stain electron photomicrographs of the supernatant and/or pellet from A-LDL fractions I and II were made and examined. The supernatant of fraction I contained unilamellar liposome-like vesicles with varying diameters, some with electron-opaque cores (Fig 2D). A small amount of cholesterol detected in the pellets of fraction I was lost during washing. The
Apo B  
Apo E  
Apo A-1  
Apo C's

**FIG 5.** Western blot analysis of sodium dodecyl sulfate (SDS) gels for apolipoprotein (apo) A-1. Apolipoproteins of plasma very-low-density lipoprotein (P-VLDL) and atherosclerotic plaque low-density lipoprotein (A-LDL) and flocculent remnants (FR particles) formed during in vitro lipolysis of hypertriglyceridemic serum were separated by SDS gradient gel electrophoresis. Left, Apo A-1 in SDS gel was probed immunologically after electrophoretic transfer of apo A-1 from the gel to a nitrocellulose membrane (right). Two lanes of A-LDL represent preparations from aortic tissues of two different donors. Details of experimental conditions are given in "Methods."

supernatant of fraction II contained electron-dense, spheroidal particles (diameter, 266±35 Å), some containing bilayer tabs (Fig 2E), that are considerably larger than plasma LDL (diameter, 210±36 Å; Fig 2C). The pellets from fraction II contained multilamellar liposome-like vesicles of varying diameters, some with aqueous cores (Fig 2F).

The major protein species of the multilamellar liposome-like vesicles were suggested by SDS gradient gel electrophoresis to be apo A-I and albumin; bands corresponding to C apoproteins also were detected, but no apo B was seen (Fig 3C). The particles were rich in unesterified cholesterol and phospholipid and poor in core lipids (Table 1).

A-LDL was further fractionated by heparin-agarose affinity column chromatography (Fig 4, top right). After removal of pelletable material from pooled LDL density fractions, A-LDL was applied to the heparin-agarose affinity column equilibrated with 0.01 mol/L Tris buffer (pH 7.4) containing 0.05 mol/L NaCl. More than 70% of the material in the A-LDL supernatant eluted without binding to the column, suggesting that these particles contained little or no apo B and/or apo E. Of the bound material, about equal amounts were eluted by 0.15 mol/L and 1.0 mol/L NaCl-Tris buffer. Control normolipidemic plasma VLDL (P-VLDL) and LDL (P-LDL) were quantitatively bound to the heparin-agarose affinity column (Fig 4, top right), but little or no control...
HDL was bound (data not shown). The P-LDL that bound to the column was largely eluted by 0.15 mol/L NaCl, but the bound VLDL required both 0.15 mol/L NaCl and 1.0 mol/L NaCl for its elution (Fig 4, top right).

Compositional analysis showed that unbound A-LDL contained more polar lipids (unesterified cholesterol and phospholipid) and less cholesteryl ester than bound A-LDL (Table 1). Unbound A-LDL was largely composed of liposome-like vesicles (Fig 4A, left); bound A-LDL consisted predominantly of spherical particles, some with tabs or vesicular blebs (Fig 4B and 4C, left). The spherical A-LDL, which bound to and eluted from 0.15 mol/L or 1.0 mol/L NaCl, contained more unesterified cholesterol and less cholesteryl ester than plasma LDL (Table 1). By SDS gradient gel electrophoresis, apo B-100 was detectable in bound A-LDL (Fig 4, bottom right, lanes B and C) but not in unbound A-LDL (Fig 4, bottom right, lane A); bands corresponding to apo A-I and C apoproteins were detectable in all fractions. The presence of apo A-I was confirmed by Western blot analysis using a monoclonal antibody to human apo A-I (Fig 5, right).

The phospholipid composition of lipoproteins from atherosclerotic plaques and plasma was examined by thin-layer chromatography. Scans of the thin-layer chromatographic plates revealed that A-VLDL and all heparin-agarose column fractions of A-LDL (Fig 6, middle) were enriched in sphingomyelin relative to phosphatidylcholine; control plasma VLDL, LDL, and HDL were enriched in phosphatidylcholine relative to sphingomyelin (Fig 6, left).

Liposome-like Particles From Human Atherosclerotic Plaques Induce Foam Cells In Vitro

We examined the ability of the multimillar liposome-like particles and apo B- and/or E-containing LDL-like particles from human atherosclerotic plaques to induce foam cells in cultured mouse peritoneal macrophages. Fig 7 shows cellular cholesterol levels of macrophages after incubation with culture medium containing plasma LDL, acetylated LDL, and extracellular liposome and lipoprotein-like particles from plaques (200 μg cholesterol per milliliter). Extracellular liposomes significantly increased the level of cellular cholesterol in-clusions over that produced by native LDL or even acetylated LDL. Liposome-like vesicles containing no apo B were two to four times more effective than apo B- and/or apo E-containing spherical LDL particles. The presence of plasma HDL in the culture dish lowered the cellular cholesterol uptake induced by the liposome-like particles.

Liposome-like Particles Produced by In Vitro Lipolysis of HTG Serum Are Similar to Those Found in Human Atherosclerotic Plaques

As noted, lipoprotein particles isolated from human atherosclerotic plaques have a number of similarities to lipolytic remnants of TG-rich lipoproteins. To subject this observation to further scrutiny, whole HTG serum was lipolyzed in vitro by the use of purified bovine milk LpL, and the characteristics of remnant lipoprotein particles produced were compared with lipoprotein-like particles isolated from human atherosclerotic plaques.

Postlipolysis HTG serum was compared with its prelipolysis control by single-spin density gradient ultracentrifugation. A new absorbance peak appeared in the postlipolysis serum on the dense side of LDL in the lipoprotein(a) density region (Fig 8, left). Material isolated from this peak was optically dense and flocculent and was pellet-free of LDL by high-speed centrifugation. This pellet material formed a discrete band when subjected to a second single-spin density gradient ultracentrifugation step (Fig 8, right, tube B). Negative-stain electron microscopy of the material from this band showed mostly large, aggregated, vesicular particles (Fig 9A); a few single liposome-like vesicles were also seen (data not shown). The lipid composition of these vesicles was predominantly FFA, phospholipid, and unesterified cholesterol with little core lipid (Table 2); protein composition was predominantly albumin, apo A-I, and C apolipoproteins with no detectable apo B (Fig 8, bottom right). The flocculent material was more clearly recognized as single or multimillar liposome-like vesicles when excess FFA was removed by washing with albumin; spherical particles with vesicular surface material were also seen (Fig 9B). Because the compositional and morphological characteristics of these particles suggest that they are derived from lipolytic surface remnants of TG-lipoproteins, we refer to them as flocculent remnants (FR particles) in the remainder of this article.

It is noteworthy that the ratios of sphingomyelin to phosphatidylcholine in the VLDL, LDL, and HDL fractions from in vitro lipolyzed HTG serum (Fig 6, right) are significantly higher than those from prelipolysis samples of HTG serum (Fig 6, left). These results
FIG 8. Characterization of liposome-like particles produced during in vitro lipolysis of hypertriglyceridemic (HTG) serum. Absorbance profiles (at 280 nm) of prelipolysis and postlipolysis aliquots of HTG serum were subjected to single-spin density gradient ultracentrifugation in an angled-head rotor (left). Photographs are shown (top right) of ultracentrifuged density gradient tubes containing HTG serum (tube A) or a band of purified flocculent remnants (FB; tube B). Sodium dodecyl sulfate gradient gel electrophoresis of very-low-density lipoprotein (VLDL), low-density lipoprotein (LDL), high-density lipoprotein (HDL), and/or the FB isolated from prelipolysis and postlipolysis samples of HTG serum (bottom right). Molecular weight (MW) standards at right. Apo indicates apolipoprotein.

HDL Dramatically Affects the Structure and Composition of Lipolytic Surface Remnants of Isolated VLDL

Because both apo A-I and C apolipoproteins are present in FR particles, we hypothesized that FR particles might represent intermediates in the movement of excess surface material from lipolyzed VLDL to HDL. To investigate this possibility, isolated VLDL was lipolyzed in vitro in the presence of varying concentrations of HDL, and the remnants produced under these conditions were characterized further.

VLDL isolated from HTG subjects was subjected to in vitro lipolysis by purified bovine milk LpL; under these conditions more than 85% of the VLDL-TG was hydrolyzed. Fig 10A shows the distribution of radioactivity after nonequilibrium density gradient ultracentrifugation of $^{125}$I-labeled VLDL lipolyzed in the absence and presence of unlabeled HDL. In the absence of HDL, lipolysis resulted in a separation of the control $^{125}$I-VLDL preparation into two peaks of radioactivity, remnant peak I in the VLDL density region and remnant peak II in the intermediate-density lipoprotein region; no label was detected in the HDL density region (Fig 10A, top). Lipolysis in the presence of a fourfold excess of HDL (cholesterol) over VLDL (cholesterol) resulted in a separation of the control $^{125}$I-VLDL preparation into two different peaks of radioactivity from that produced in the absence of HDL (Fig 10A, bottom). One of the peaks (remnant peak III) is intermediate-density lipoprotein in density but shifted toward higher values from that of peak II; the second peak (remnant peak IV) is in the HDL density region.

Negative-stain electron microscopy of remnant material from peaks I and II produced in the absence of HDL showed core-depleted particles to which excess vesicular surface remnants were still attached (Fig 10B-2); these remnants included all of the C apoproteins of control VLDL (Fig 10C, lane 2). These VLDL remnant particles are structurally and compositionally similar to VLDL and LDL-like particles isolated from human atherosclerotic plaques (compare Figs 2 and 3).
with Fig 10). Analysis of remnant material from peak III produced in the presence of HDL showed electron-dense spherical particles without detectable vesicular surface material and essentially devoid of C apoproteins (Figs 10B-3 and 10C, lane 3). Further analysis of this core remnant material showed that apo A-I, not present in remnants produced in the absence of HDL (Fig 10C, lane 2) or in control VLDL (data not shown), had moved to remnant peak III from HDL (Fig 10C, lane 3). A measurable but much smaller amount of apo A-I was transferred from HDL to VLDL during incubation of control VLDL with HDL in the absence of LpL (Fig 10C, lane 1). It is important to note that after lipolysis in the presence of HDL, remnant HDL density particles located in peak IV were larger than before lipolysis but remained spheroidal (Fig 10B-4 and 10B-5).

To pursue further our hypothesis that FR particles might represent intermediates in the movement of excess surface material from lipolyzed VLDL to HDL, VLDL lipolyzed in vitro in the presence of albumin alone was incubated with increasing concentrations of HDL. As shown in Fig 11, in the absence of HDL no FR particles were seen; FR particles first appeared at an HDL-to-VLDL molar ratio of 0.25:1. As the HDL-to-VLDL molar ratio increased to 2:1, the concentration of the FR particle band increased until it suddenly disappeared at a molar ratio of 4:1, to be replaced by a nonflocculent HDL density band.

These results suggest that FR particles represent a coalescence of VLDL surface remnants, including phospholipid, unesterified cholesterol, and C apolipoproteins, with free apo A-I or intact apo A-I–enriched HDL particles. This conclusion is based on the following observations: (1) unlipolyzed control VLDL contains no apo A-I, (2) VLDL lipolyzed in vitro in the absence of HDL produces spheroidal particles containing surface vesicular remnants and all of the starting C apolipoproteins, and (3) the FR particles are enriched in apo A-I and C apolipoproteins, as well as phospholipids and unesterified cholesterol. This model is compatible with a previous suggestion by Tall and Small33 that apo A-I may play a role in dissociating lipolytic surface remnants from core remnants of TG lipoproteins.

**Discussion**

Liposome-like particles appear in the extracellular space of the arterial subendothelium during the pre–foam cell stages of atherosclerosis in experimental animals. This fact, together with our observation that liposome-like particles from human atherosclerotic plaques are powerful inducers of macrophage/foam cells in vitro, suggests that accumulation of liposome-like particles in the arterial wall represents a direct step toward foam cell formation in vivo. Although the origin of liposome-like particles in human atherosclerotic plaques is much more complex than those extracellular...
liposome-like vesicles found in the pre–foam cell lesions of experimental animals, we see three possible (not necessarily exclusive) ways that the liposome-like particles containing apo A-I and C apolipoproteins and enriched in sphingomyelin could have arisen. (1) The liposome-like particles represent LDL or β-VLDL entrapped in the intimal space and subsequently modified.4 Perhaps the apo A-I and C apolipoproteins have been displaced or otherwise transferred from HDL particles in flux from the plasma compartment through the intima. (2) The liposome-like particles have a cellular origin, such as lipid material extruded from necrotic or otherwise damaged arterial wall cells13,14 and/or secreted from cholesterol-enriched cells,34 such as foam cells and endothelial cells. This possibility can explain the sphingomyelin enrichment of these particles.15 (3) The liposome-like particles represent lipolytic surface remnants of TG-rich lipoproteins produced in the intimal space by LpL secreted from arterial wall cells35-36 after entrapment of intact TG-rich lipoproteins and/or produced in the plasma compartment as part of the normal lipolytic process.

Nievelstein et al11 have demonstrated that a bolus infusion of human plasma LDL into rabbits, resulting in a 5- to 20-fold increase in plasma LDL levels, causes deposition of LDL-size lipid particles in the subendo-
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Fra 11. Photographs showing effect of high-density lipoprotein (HDL) concentration on appearance or disappearance of flocculent remnant (FR) particles after incubation of prelipolyzed very-low-density lipoprotein (VLDL) with HDL. Increasing concentrations of control HDL were added to a constant amount of VLDL prelipotyzed in the presence of albumin alone; VLDL to HDL cholesterol ratios were 1:0, 1:0.25, 1:0.5, 1:1, 1:2, and 1:4. The mixtures were incubated at 37°C for 4 hours and subjected to single vertical-spin density gradient ultracentrifugation in a Beckman SW 41 swing-out rotor (procedure described in detail in the legend to Fig 1).

...the intimal space. These LDL-size lipid particles are often grouped together in grapelike clusters. Clustering and fusion of LDL or β-VLDL entrapped in the intimal space may explain some properties of the liposome-like particles in atherosclerotic plaques. This is probably the most widely accepted hypothesis to explain the presence of liposome-like vesicles. However, the physicochemical mechanisms for this putative process remain unexplained. Furthermore, the sphingomyelin enrichment in liposome-like vesicles in atherosclerotic plaques cannot be accounted for by aggregation and fusion of otherwise intact LDL or β-VLDL particles.

While there may be a partial role for intracellular processing and cell degeneration in explaining the origin of liposome-like particles, the marked similarity of particles to lipolytic remnants of TG-rich lipoproteins and the presence of apo A-I and C apolipoproteins cannot easily be explained by this hypothesis alone.

The structural similarity of liposome-like lipid particles to partially lipolysed TG-rich lipoproteins and to FR particles produced in vitro lipolysis of HTG serum is striking. The relative enrichment of sphingomyelin over phosphatidylcholine in these particles can be explained by the phospholipase activity of LpL, which, as the in vitro lipolysis experiments show, selectively lowers the level of phosphatidylcholine relative to sphingomyelin in TG-rich lipoprotein remnants. The relative enrichment of cholesterol over TG in these particles would be expected for any TG-rich lipoprotein particle trapped in the artery wall, since LpL on arterial wall cells will hydrolyze TG to FFA for removal by cells and/or albumin. However, no mechanism is known for the removal of cholesterol from the subendothelial space of the artery wall.

These results emphasize the possibility that the origins of liposome-like particles in atherosclerotic plaques may involve some form of lipolysis. The initial substrate for this lipolysis could be cholesterol-rich lipoproteins, such as cholesterol-rich VLDL, LDL, or β-VLDL. This may explain the enrichment in sphingomyelin but does not easily explain the presence of apo A-I or the liposome-like vesicles. The possibility that liposome-like particles represent lipolytic surface remnants of TG-rich lipoproteins seems more compatible with our observations. LpL has been shown to be produced by arterial smooth muscle cells and macrophages in human atherosclerotic lesions and an atherogenic role for lipolysis in the arterial wall has been proposed. The concept that the liposome-like particles may be the products of lipolysis of intact, matrix-entrapped, TG-rich lipoproteins, therefore, is not a radical concept. On
the other hand, the concept that these particles may be produced in the plasma compartment as part of a normal lipolytic process gone awry is novel and requires some justification.

One legitimate concern is whether surface remnants of TG-rich lipoproteins have a “finite” lifetime in vivo. It is generally believed that lipolytic surface remnants of TG-rich lipoproteins produced after lipolysis in vivo are taken up by circulating plasma HDL. However, Forte et al have shown that heparin-induced lipolysis of plasma from HTG subjects results in the accumulation of FFAs, liposome-like vesicles and large spherical particles with lamellar structure on the surface. Tall et al have shown that in vivo lipolysis of TG-rich lipoproteins in rats after an injection of a large dose of chylomicrons results in the appearance of liposome-like vesicles in blood. These in vivo data suggest that free lipolytic surface remnants and/or surface remnant-enriched remnants of TG-rich lipoproteins may exist under certain circumstances in circulating blood, eg, in dyslipidemic subjects or during the postprandial lipemic state.

A second issue to consider concerns flow dynamics and sites of lipolysis of TG-rich lipoproteins. Lipolysis of TG-rich lipoproteins occurs predominately at LpL binding sites on the endothelial surface of blood vessels. Not much is known about the density of LpL binding sites on the endothelial surface of large to medium-size arteries, where atherosclerotic plaques occur, although such binding sites are known to be present. Nevertheless, a likely possibility is that an episodic concentration gradient of potentially atherogenic TG-rich lipoprotein remnants exists at the endothelial surface of these arteries. Under these conditions and as a result of lamellar flow dynamics, the remnant-to-HDL ratio at the endothelial surface would be expected to be much higher than in the circulation. It is important to note that endothelial cells have a higher concentration of the putative “HDL receptor” than any other cell type examined. Perhaps one function of the HDL receptor is to raise the HDL concentration at the endothelial cell surface to match that of the potentially atherogenic TG-rich lipoprotein remnants.

The results presented here show that apo A-I is a major apolipoprotein component of liposome and lipoprotein-like particles in atherosclerotic plaques. It is important to understand the origin of this apo A-I. Our in vitro lipolysis results show that liposome-like vesicles, similar to liposome-like particles in atherosclerotic plaques, are formed during in vitro lipolysis of VLDL in the presence of low concentrations of HDL or on incubation of lipolyzed VLDL with low levels of HDL. The apo A-I and apo C on liposome-like vesicles are most likely derived from the HDL and VLDL remnants, respectively. Musliner et al have reported similar observations. They have shown that lipolysis products of VLDL, predominantly FFAs, induce aggregation of VLDL with LDL in vitro. Increasing concentrations of HDL progressively inhibit this complex formation and result in the association of HDL-derived apo A-I with VLDL and LDL after lipolysis. Very little apo A-I association occurs in the absence of lipolysis.

Our in vitro studies together with those of Musliner et al suggest that one or more specific HDL subspecies lose some or all of their apo A-I to excess surface remnants (perhaps through particle-particle interactions) to produce the FR particles; part of the driving force for this movement of apo A-I may be an elevated concentration of FFAs on remnant surfaces. A reasonable working model (Fig 12) is that during lipolysis of TG-rich lipoproteins, apo A-I from a certain HDL subspecies is involved in dissociating surface remnants from the core remnants of TG-rich lipoproteins. Under normal conditions these dissociated surface remnants (FR particles), which represent a normally short-lived intermediate, are cleared further by the same or other subspecies of HDL, resulting in the production of new apo C-rich HDL subspecies.

What are the possible implications of this working model for the origin of liposome-like particles in atherosclerotic plaques? One possible scenario is that HDL deficiency results in a buildup of intermediate products of lipolysis, such as FR particles and/or core remnant particles containing excess surface remnant components, at the site of lipolysis (endothelial surface and/or close proximity to foam cell lesions). A number of in vitro studies have shown that the presence of excess surface components (C apolipoproteins) on apo B-and/or apo E-containing lipoproteins inhibits the binding of these lipoproteins to the LDL receptors of extrahepatic tissue. Either or both of these abnormal remnant particles might be prone to cross the endothelial barrier and/or become entrapped in the arterial intima to produce the liposome and liposome-like extracellular particles associated with atherosclerotic plaques (Fig 12). It has been shown previously by us that FFA-enriched lipolytic remnants of TG-rich lipoproteins produced after in vitro lipolysis of HTG serum can disrupt the endothelial barrier function and thus allow transfer of macromolecules across the endothelial monolayer. Alternatively, deficient HDL might be ineffective in interacting with atherogenic lipoprotein-like vesicles in atherosclerotic plaques and thus fail to assimilate them for removal from the arterial wall. We propose as a working hypothesis that one role of HDL in inhibiting the atherogenic process is to assimilate the highly atherogenic liposome-like particles (1) in a "mop-up" fashion to remove them from the artery wall and/or (2) in a preventive fashion in the plasma compartment to inhibit their formation and transcytosis into the arterial wall.

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Liposome-like particles isolated from human atherosclerotic plaques are structurally and compositionally similar to surface remnants of triglyceride-rich lipoproteins.

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