Lipoprotein-like Particles and Cholesteryl Esters in Human Bruch’s Membrane: Initial Characterization

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**PURPOSE.** To isolate and characterize cholesteryl ester-containing, lipoprotein-like particles (LLPs) from normal aged human Bruch’s membrane (BrM)/choroid (Ch).

**METHODS.** From BrM/Ch of 20 eyes of 10 donors aged >60 years, LLPs were isolated using high-salt buffer, fractionated by density gradient ultracentrifugation, and characterized by determining cholesterol, triglyceride, and phospholipid concentration; by enzymatic colorimetry and fluorometry; by cholesteryl ester composition; by electron microscopy (negative stain electron microscopy). Apolipoprotein (apo) gene expression was determined by RT-PCR, Western blot analysis, and immunofluorescence of retinal–choroidal cryosections. In paraformaldehyde-preserved eyes (20 eyes of 20 donors), cholesteryl ester composition of BrM/Ch, cornea, and sclera was determined by ESI/MS.

**RESULTS.** A pooled fraction of LLP released from BrM/Ch (concentrated total LLP, density [d] < 1.24 g/mL fraction) was fractionated into two peaks. A large Peak 1 (with plasma LDL-HDL density range), containing predominantly phospholipid and unesterified cholesterol, was morphologically heterogeneous. A small Peak 2 (with plasma VLDL density range), enriched with esterified cholesterol, contained ~100 nm diameter round electron-lucent particles. Both peaks contained apoB and apoA-I, RPE and retina contained apoA-I transcripts, and BrM and drusen contained apoA-I immunoreactivity. Peaks 1 and 2, native RPE, and fresh BrM/Ch were cholesteryl linolate enriched and contained little cholesteryl docosahexaenoate. Preserved BrM/Ch was cholesteryl oleate-enriched, unlike sclera and cornea.

**CONCLUSIONS.** BrM/Ch LLP do not resemble plasma lipoproteins in density profile, cholesterol distribution, or morphology. Peak 2 contains EC-rich LLP resembling BrM particles in situ. BrM/Ch cholesteryl esters respond to long-term storage differently than esters of plasma lipoprotein origin accumulated in other ocular tissues. Evidence of intracellular apoB and apoA-I expression supports an emerging hypothesis that the RPE assembles and secretes a large, possibly novel, lipoprotein particle. (Invest Ophthalmol Vis Sci. 2005;46:2576–2586) DOI:10.1167/iovs.05-0034

A ge-related maculopathy (ARM) is the leading cause of new, untreatable vision loss in the elderly of industrialized nations.1 A heterogeneous disorder, ARM’s most prominent clinical and histopathologic lesions affect the retinal pigment epithelium (RPE), Bruch’s membrane (BrM), and the choriocapillaris, ultimately affecting the function of the photoreceptors.2,3 Early ARM is characterized by drusen and/or pigmentary changes. Late ARM is characterized by geographic atrophy and/or choroidal neovascularization and its sequelae. Limited treatment options for ARM are directed either against choroidal neovascularization, a sight-threatening complication in late ARM, or entail providing antioxidant nutritional supplements for some patients with early ARM. Valuable new information has emerged about molecules within drusen (characteristic, focal extracellular lesions), including inflammation-associated proteins and advanced glycation end products.4,5 Yet underlying mechanisms leading to drusen and basal linear deposit (drusenoid material in a diffusely distributed lesion),6 remain obscure.

Lipoproteins are multimolecular assemblies with neutral lipid cores of triglyceride (TG) and esterified cholesterol (EC) surrounded by a solubilizing surface of apolipoprotein (apo), phospholipid (PL), and unesterified cholesterol (UC).7 Classically defined major lipoprotein classes include (but are not restricted to) high-density, low-density, and very-low-density lipoproteins and dietary chylomicrons (HDL, LDL, VLDL, and CM, respectively). Lipoprotein particles differ in size, flotation properties in a density gradient, electrophoretic mobility, ratio of lipid to protein, ratio of TG to EC in the core, and major surface apo. Lipoproteins containing apoB (hepatic VLDL and its metabolite LDL, and intestinal CM and partly hydrolyzed CM remnants) are considered atherogenic, as they penetrate vascular intima before development of frank lesions.8,9 The component apop classify lipoprotein particles most specifically,10 and in humans, VLDL particles contain apoB-100, apoE, and apo C-I, -II, -III; LDL contains apoB-100 only; and CM particles contain apoB-48, apo C-I to -III, and apoA-I.
A growing body of literature indicates the involvement of lipids and lipoproteins in the formation of the characteristic extracellular lesions in aging and ARM eyes. Neutral lipids, including EC and UC, are histochemically detectable in drusen. Material binding oil red O (a neutral fat stain) and containing EC and TG accumulates in normal BrM throughout adulthood, suggesting a large, universal, and constitutive yet poorly understood process of lipid deposition. That lipoprotein particles are a potential means of depositing neutral lipids is indicated by immunohistochemical localization of apoB and apoE in drusen and ultrastructural visualization of solid 100-nm diameter particles with a surface and core substructure in BrM- and ARM-associated lesions. The probability that a culprit particle is of intraocular origin is increased by evidence that native human RPE expresses mRNA transcripts for apoE, apoB, and microsomal triglyceride transfer protein, required for assembly of an apoB-containing lipoprotein and the product of the abetalipoproteinemia gene (Menckelian Inheritance in Man [MIM] 2001001; http://www.ncbi.nlm.nih.gov/Omim/ provided in the public domain by the National Center for Biotechnology Information, Bethesda, MD). Further, cultured native RPE and ARPE-19 cells secrete apoE and neutral lipid, respectively.

The potential role that lipoproteins play in ARM would be clarified by evidence that lipoprotein-like particles (LLPs) can be isolated from BrM/Ch, as they are from atherosclerotic arterial intimas. In the current study, we showed that double, high-salt buffer extraction can release such particles for initial characterization of protein and lipid composition. Special attention was directed to EC, localized exclusively within BrM by specific histochemistry. Because BrM/Ch preparations unavoidably retain some plasma lipoproteins of hepatic and intestinal origin, we compared our isolates to plasma lipoproteins. In light of the evidence of intraocular apoB and apoE expression, we also sought evidence of apoA-I expression. Finally, we analyzed EC composition in BrM and other ocular regions in fresh and preserved eyes, finding evidence that BrM EC may be uniquely susceptible to modification in storage. These findings collectively suggest that BrM/Ch LLPs do not resemble plasma lipoproteins in several key attributes.

**Methods**

**Human Plasma Lipoproteins**

To obtain plasma lipoproteins, fresh plasma from fasting and nonfasting normolipidemic volunteers was adjusted to density (d) = 1.24 g/mL using KBr and then centrifuged at 49,000 rpm for 36 hours at 4°C in a swing-out rotor (SW55; Beckman Instruments, Fullerton, CA). Lipoprotein mixtures were collected by removing the top 1-mL fraction from the centrifuge tubes. Density gradients were then formed by placing 1.0-mL aliquots of these mixtures at the bottom of a 5-mL density gradient tube containing 2.5 mL Tris-buffered saline (TBS; 0.01 Tris and 0.15 M NaCl [pH 7.4]) at the top and 1.5 mL of a d = 1.12 g/mL KBr solution at the bottom. After ultracentrifugation at 49,000 rpm for 150 minutes at 4°C, VLDL, banded at the top; LDL, banded at the upper middle; and HDL, retained at the bottom of the tubes, were collected by aspirating visible lipoprotein bands from the tube tops. These fractionated lipoprotein samples were dialyzed against TBS for 24 hours to remove KBr. To separate any CM recovered in the VLDL density fraction, a dialyzed VLDL density sample (1 mL) was placed under 4 mL buffered saline in a centrifuge tube and subjected to brief (30 minutes) ultracentrifugation at 30,000 rpm. CM floated to the tube tops. VLDL at the tube bottoms were then collected.

**Human Ocular Tissues**

Human eyes were obtained from nondiabetic donors within 6 hours of death through the Alabama Eye Bank. Eyes from only those donors ≥ 60 years old, with high expected BrM lipid content, were used. Globes were inspected internally with epi- and transillumination. Fresh eyes were used for isolating RPE and BrM/Ch LLPs. Eyes preserved by immersion in 4% paraformaldehyde and stored in 1% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4) at 4°C (mean storage time, 573 ± 235 days) were used for EC compositional analysis and immunohistochemistry. Human tissue use was approved by institutional review (protocol X010705001), and the protocol adhered to the guidelines set forth by the Declaration of Helsinki.

**Particle Isolation from Human BrM**

Fresh globes were incised circumferentially at the equator, penetrating the sclera, retina, and vitreous. The neurosensory retina and vitreous body were removed together after excision of retina at the optic nerve head. The resultant eyebcup was rinsed twice with 1 mL chilled isolation buffer (0.01 M Tris, 0.15 M NaCl, 0.02 mM butylated hydroxytoluene, and 0.1% EDTA, and 0.01% NaN₃ [pH 7.4]). Then, 500 μL buffer was twice added to the eyecup, and the RPE was brushed lightly from the optic nerve outward under a dissecting microscope. Dislodged RPE cells were collected, resuspended in 1 mL of PBS, and used for lipid extraction and mRNA isolation. The cells in 0.1 mL volume were digested in 0.1 N NaOH/0.1% sodium dodecyl sulfate (SDS) to determine protein content (DC Protein Assay Kit; Bio-Rad, Hercules, CA). One milliliter buffer was added to the eyecup to clear the remaining RPE and then discarded. Then, BrM, recognizable by its translucent white polka-dotted appearance, and the attached choroid were removed and placed in isolation buffer. After five rinses, large choroidal vessels were removed under the dissecting microscope. Twice, blood was milked out of the remaining large vessels by brushing gently from the anterior margin to the optic nerve head. The resultant BrM/Ch preparation was stored in 1 mL isolation buffer with protease inhibitor at 4°C until particle isolation.

BrM/Ch was homogenized in 3 mL isolation buffer with a glass homogenizer until large clumps disappeared. After the homogenate was spun at 10,000 rpm for 6 minutes, solid tissues were pelleted, and the supernatant was transferred to a new 5-mL tube. To release any lipoproteins bound to tissue proteoglycans, pellets were resuspended in 1 mL of isolation buffer containing 1 M NaCl and placed in a bath sonicator for 5 to 10 minutes. After samples were spun at 10,000 rpm for 6 minutes, the second supernatant was pooled with the first. Lipoproteins were isolated by adjusting the density of the pooled buffer extract to d = 1.24 g/mL KBr and subsequent ultracentrifugation at 49,000 rpm for 36 hours with a swing-out rotor (SW55; Beckman). BrM/Ch LLPs, collected by withdrawing the top 1-mL fraction from the ultracentrifuge tubes, was then subjected to density gradient ultracentrifugation as described for plasma lipoproteins. After ultracentrifugation, BrM/Ch LLPs in the density gradient tubes were separated into 19 subfractions by puncturing the tubes with a density gradient fractionator (Beckman). Lipoprotein peaks were located in the gradient by using an enzymatic fluorometric assay to define a cholesterol profile (described later). On the basis of this profile, density gradient fractions containing major peaks were pooled and dialyzed with pH 7.4 Tris-HCl buffer for 4 hours. Aliquots were subjected to colorimetric enzymatic assays for lipids using cholesterol and phospholipid enzymatic assay kits from Waco Diagnostic Co. (Richmond, VA), lipid extraction, or Western blot analysis. Preliminary experiments indicated that the fluorometric and colorimetric cholesterol assays gave a similar three-peak distribution for plasma lipoproteins, with 50% to 80% higher cholesterol detected by fluorometry. This higher sensitivity was required for the 19 small subfractions but not the large pooled fraction of BrM/Ch lipoproteins.

Concentrations of total cholesterol (TC), TG, and PL determined by colorimetric enzymatic assays were expressed as milligrams per deciliter and converted to nanomoles using molecular weights for cholesterol, cholesteryl oleate (for EC), triolein (for TG), and phosphatidyl-
cholesteryl oleate; 18:2, cholesteryl linoleate; 20:4, cholesteryl arachidonate; 22:6, cholesteryl docosahexaenoate.

Cholesterol Mass and EC Composition: Sample Preparation and Assays

Lipids were extracted from tissues, cells, and lipoproteins with 7.0 mL chloroform, 3.5 mL methanol, and 0.5 mL water. Extracts were left to stand for 15 minutes, and 2.5 mL water was added and left to stand for 2 hours while phases separated. Upper (aqueous) and middle (proteinaceous) phases were discarded. After the bottom (organic) phase was washed with methanol-chloroform-water (48:3:4), three 500-µL aliquots were evaporated under nitrogen and dried in a vacuum. Aliquots were solubilized in isopropanol (50 µL for BrM, retina, RPE, LDL, and macrophages; 500 µL for cornea and sclera), vortexed, and sonicated for 15 minutes.

TC and UC in extracts of native RPE, BrM/Ch LLP, and plasma lipoproteins was determined by an enzymatic fluorometric assay as described for 100-, 50-, and 2-µL aliquots of each preparation.

EC concentration was defined as the difference between TC and UC in extracts of native RPE, BrM/Ch LLP, and plasma lipoproteins. UC dissolved in isopropanol was a standard. Cholesterol ester composition was assayed by electrospray ionization mass spectrometry (ESI/MS) as described, using isopropanol-d8 as standard. Cholesteryl ester composition was also assayed in partially isolated BrM and other parts of paraformaldehyde-preserved eyes. These tissues were used because of availability and because EC had been extracted and assayed from similar tissues previously. Eight-millimeter diameter punches of macular retina, RPE, choroid, and sclera were obtained and separated as described. Examination of removed RPE by electron microscopy indicated cell sheets with some basal laminar deposit (not shown). For cornea, the central 8 mm was removed with a trephine, leaving a ring of corneal periphery with arcus lipoides, a benign, age-related EC accumulation. The effect of paraformaldehyde fixation was investigated by comparing ocular EC composition in two pairs of fellow eyes. An eye fixed for 7 days was compared to a fellow eye that was dissected fresh and extracted immediately. In another pair, one eye was preserved and stored in paraformaldehyde, and the fellow eye was frozen fresh and stored at −80°C. Both eyes were dissected and extracted after 3 months. Cholesterol ester composition was also assayed in LDL (20 µL, 5 mg/mL) isolated from healthy subjects by ultracentrifugation and acetylated LDL (50 µL, 2.5 mg/mL) used to load cultured macrophages. Results from LDL and acetylated LDL were similar and therefore combined. Human monocyte macrophages were isolated, cultured, exposed to acetylated LDL to induce large intracellular EC-rich droplets, and extracted as described earlier.

**Cholesterol Mass and EC Composition:**

The mass of cholesterol and its esterification in different tissues can be quantified. The Cholesterol Mass and EC Composition section describes the extraction and quantification of cholesterol and its esterification in different tissues. This includes the analysis of cholesterol in native RPE, BrM/Ch LLP, and plasma lipoproteins.

**Reverse Transcriptase–Polymerase Chain Reaction:**

Total RNA was isolated from neurosensory retina, native RPE, ARPE-19, and HepG2 cells. The following primer sequences for RT-PCR were designed: apoA-I (sense, 5'-AAG ATG ACC CCC AGA G-3', antisense; 5'-TTC AGC TTC AGC AGC G-3'), lecithin cholesterol acyl transferase (LCAT, sense, 5'-ATTCAA TGT GCT CTT CCT C-3', antisense, 5'-GCT TGC GGT AGT ACT CCT C-3'). To distinguish between amplified mRNA and genomic DNA, all primers were designed to span intron boundaries. One-step RT-PCR was used with kits (Qiagen, Valencia, CA). Reverse transcription was performed at 52°C for 30 minutes followed by 15 minutes at 95°C to inactivate the reverse transcriptase and activate the DNA polymerase (HotStarTag; Bio-Rad). The reaction was amplified through 30 cycles, each consisting of 30 seconds at 94°C (denaturing), 30 seconds at 60°C (annealing), and 1 minute at 72°C (extension). The reaction was incubated at 72°C for another 10 minutes. RT-PCR products were resolved on 1% agarose gel, stained with ethidium bromide, and visualized by ultraviolet transillumination. Expected sizes of RT-PCR products are 679 bp (apoA-I) and 470 bp (LCAT).

**Western Blot Analysis:**

**ApoB.** Aliquots of BrM/Ch LLP were concentrated approximately 15-fold (Centricon YM-10; Millipore, Bedford, MA) and separated by 4% to 12% SDS-polyacrylamide gel electrophoresis (SDS-PAGE). After electrophoresis, proteins were transferred onto polyvinylidene fluoride membranes by Laemmli's transfer buffer system and detected by Western blot analysis with biotinylated antibody to human apoB-100, as described.

**ApoA-I.** Aliquots of BrM/Ch LLP were subjected to SDS-PAGE on 12% or 16% Tris-glycine gels. Proteins were transferred to a nitrocellulose membrane. The membrane was blocked in 3% gelatin in TBS for 1 hour at room temperature and incubated with biotinylated affinity-purified polyclonal goat anti-human apoA-I antibody (Brookwood Medical, Birmingham AL) overnight at 4°C. After a thorough TBS wash, the membrane was incubated with streptavidin-alkaline phosphatase conjugate (Bio-Rad) for 1 hour at room temperature. ApoA-I immunoreactivity was developed by an alkaline phosphatase conjugate substrate kit (Bio-Rad) according to the manufacturer’s protocol. Purified human plasma apoA-I served as a positive control.

**Reactor Culture**

ARPE-19 and HepG2 cell lines were obtained from the American Type Culture Collection (Manassas, VA). ARPE-19 cells of passage 22 were grown for 4 weeks in T-75 flasks or six-well plates in DMEM/F12 (1:1) containing 10% fetal calf serum (FCS) as described. Medium was changed twice weekly. HepG2 cells were grown for 5 days in MEM containing 10% FCS in six-well plates with medium changed every other day. All cells were cultured at 37°C and 5% CO₂.

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Negative Stain Electron Microscopy

LLPs were visualized as described. Samples were dialyzed in 0.26 mM EDTA, 2.6 mM ammonium carbonate, and 0.125 M ammonium acetate at 4°C for 12 hours (Slide-A-Lizer Dialysis Cassettes; Pierce, Rockford, IL). Two microliters of dialyzed sample was placed on 200-mesh copper grids with polyvinyl film (Formvar; SPL, West Chester, PA). Carbon support film freshly decharged with 100% ethyl alcohol. Grids were stained for 30 seconds with 2 μL of 2% potassium phosphotungstic acid solution extruded through a filter with 0.2-μm-diameter pores. Excess moisture was wicked with filter paper wedges and the grids allowed to dry. Comparison grids contained only dialyzing buffer. Samples were viewed on a microscope (JEM-1200EX II; JEOL, Tokyo, Japan; accelerating voltage 60 KV) and photographed (4889 film; Kodak, Rochester, NY). Negatives were scanned (model 1100 Power-Look scanner and Umax Magiscan 4.5; Umax Technology, Milton Keynes, UK) at 600 ppi, printed at 2.87 magnification, and imported into image-manipulation software (Photoshop 7.0; Adobe Systems, Mountain View, CA) for contrast enhancement and compositing. Electron microscopy supplies came from EMS (Fort Washington, PA).

Indirect Immunofluorescence of Apos

Cryosections of human retina and choroid were processed for apoA-I immunoreactivity as described, with a polyclonal antibody to apoA-I (catalog number K45252G; Biodesign, Saco, ME), and nonimmune rabbit immunoglobulin at an equivalent protein concentration was used for the control. Sections were viewed by microscope (Optiphot 2; Nikon, Melville, NY), epifluorescence optics (excitation, 546 nm; emission, 590 nm), and a dry 40 × planapochromat objective (numeric aperture [NA] = 0.95). Photographs were then taken (Delta 100 film; Ilford Photograph Corp., Paramus, NJ) with matched exposure times for experimental and control sections. Images were scanned from negatives (SprintScan 4000; Polaroid Corp., Cambridge, MA) and assembled into composite photomicrographs (Photoshop; Adobe Systems).

RESULTS

Cholesterol-Containing Particles in BrM/Choroid

To define the cholesterol profile of BrM/Ch LLP, we subjected concentrated buffer extracts containing all lipoproteins (d ≤ 1.24 g/mL fraction, herein called the pooled fraction) from seven pairs of eyes to density gradient ultracentrifugation and obtained 19 subfractions. In contrast to the three clearly defined plasma peaks (Fig. 1A), the d ≤ 1.24 g/mL fraction of all BrM/Ch preparations contained two peaks (Fig. 1B). A large Peak 1 located in the plasma LDL-HDL range (fractions 3–13) was low in EC (EC/TC molar ratio = 0.14). A small Peak 2 located in the plasma VLDL range (fractions 16–19) was rich in EC (EC/TC molar ratio = 0.54). In different eyes, the ratio of TC recovered from Peak 1 to TC recovered from Peak 2 ranged from 2.63 (donor 4) to 25.96 (donor 6). Thus, cholesterol from Peak 1 dominated the pooled fraction.

A more detailed characterization of lipids in the pooled fraction and in Peaks 1 and 2 is shown in Table 2. Regarding the pooled fraction, the total amount of cholesterol recovered was highly variable among eyes. The coefficient of variation (SD/mean) ranged from 39% to 53% for each of the measured lipids. However, each lipid’s proportion of the total was remarkably consistent across the same eyes, with coefficients of variations ranging from 4.5% to 18.5%. Mean proportions of PL, EC, UC, and TG mass measured in eight donors was 0.38, 0.20, 0.57, and 0.06, respectively. Overall, the proportion of TC that was esterified was 0.35 ± 0.04. Because a different number of subfractions comprised Peaks 1 and 2, more weight should be given to molar ratios of lipids within peaks than measured concentrations. Regarding Peak 1, sufficient material was available for analysis by colorimetric assay in seven donors. In these samples, PL, EC, UC, and TG represented mean mass proportions of 0.43, 0.10, 0.41, and 0.05, respectively, of the lipids measured in Peak 1. The proportion of TC that was esterified was 0.20 ± 0.07, and the ratio of UC to PL, which solubilizes UC, was 0.95 ± 0.14. Regarding Peak 2, no lipids except cholesterol could be assayed due to the small sample size. At 0.59 ± 0.15, the proportion of TC that was esterified in Peak 2 was two times higher than Peak 1. TG was measurable but not abundant in the pooled fraction and in Peak 1 (pooled fraction, TG/EC = 0.31 ± 0.06; Peak 1 = 0.64 ± 0.31). For comparison plasma TG/EC for the HDL, LDL, and VLDL peaks of a normolipemic subject are 0.09, 0.26, and 14.2, respectively.

To characterize the EC composition of BrM/Ch LLP, extracts of the pooled fraction, Peak 1, and Peak 2 were assayed by ESIMS and the concentrations normalized to cholesteryl oleate (18:1; Fig. 2). In these fractions, cholesteryl linoleate (18:2) was the predominant ester, being 2.1-, 2.7-, and 2.5-fold more abundant, respectively, than cholesteryl oleate. The cholesteryl palmitate (16:0) concentration was similar to that of cholesteryl oleate. In contrast, little cholesteryl stearate (18:0), cholesteryl arachidonate (20:4), or cholesteryl docosahexaenoate (22:6) was found in the pooled fraction (normalized con-
centrations of 0.56, 0.14, and 0.12, respectively), Peak 1 (0.092, 0.157, 0.008), or Peak 2 (0.099, 0.149, 0.012). These proportions were not dissimilar from plasma lipoproteins asayed by this method. For example, LDL from a normolipemic subject has 2.2-fold more cholesteryl linoleate than cholesteryl oleate and normalized cholesterol docosahexaenoate concentrations of only 0.01 (Fig. 2A). Of the three plasma lipoproteins examined, the EC composition of Peaks 1 and 2 resembled LDL (0.092, 0.157, 0.008), or Peak 2 (0.099, 0.149, 0.012). These concentrations of 0.56, 0.14, and 0.12, respectively), Peak 1 (0.092, 0.157, 0.008), or Peak 2 (0.099, 0.149, 0.012). These proportions were not dissimilar from plasma lipoproteins asayed by this method. For example, LDL from a normolipemic subject has 2.2-fold more cholesteryl linoleate than cholesteryl oleate and normalized cholesterol docosahexaenoate concentration of only 0.01 (Fig. 2A). Of the three plasma lipoproteins examined, the EC composition of Peaks 1 and 2 resembled LDL most closely and CM least (Fig. 2B).

To determine whether BrM/Ch cholesterol is associated with classic plasma lipoprotein particles, pooled fractions, Peak 1, and Peak 2 from seven eyes were subjected to Western blot analysis. ApoB-100 bands and a lower molecular weight band appeared in Peaks 1 and 2 (Fig. 4A). A strong band at 28 kDa for apoA-I appeared in the individual subfractions comprising Peaks 1 and 2 (e.g., Fig. 4B; lanes 4–7). Other bands were detected at 56 kDa in lanes 2, 3, 4, and 5 and at 84 kDa in lane 5 only. Of the seven eyes analyzed, apoB-100 was detected in four, and apoA-I was detected in seven. ApoA-I was also detected in human retina and native RPE (Fig. 4C, lanes 3, 4).

To determine whether Retina and neurosensory retina express mRNA transcripts for apoA-I, we performed RT-PCR using total mRNA isolated from retina, native RPE, ARPE-19 cells, and HepG2 hepatoma cells. The expected 679-bp PCR product was detected in each sample (Fig. 5A). In plasma, apoA-I activates the cholesterol-esterifying enzyme lecithin cholesterol acyl transferase (EC 2.3.1.43, LCAT40). LCAT is bound to HDL and transferred to apoB-containing lipoproteins by cholesterol ester transfer protein. Therefore, we determined whether retina and RPE also express mRNA for LCAT. Once again, the expected PCR product (470 bp) appeared in each sample (Fig. 5B).

### Table 2. Characterization of Lipids in Pooled d ≤ 1.24 g/mL Fraction, Peak 1, and Peak 2

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<tr>
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Except TC and UC of Peak 2 detected by enzymatic fluorimetry, all others were detected by enzymatic colorimetry. —, not done.

* Molecular weight used: UC, 386.7; TG, 885.4; PL, 760.1; EC, 651.1.

† Expressed relative to (TC+TG+PL).
To localize apoA-I in normal human retina and choroid, we probed cryosections with polyclonal antibodies to human apoA-I and detected immunoreactivity by indirect immunofluorescence. Sections of normal peripheral retina contain intense labeling in BrM and drusen and less intense, diffuse labeling within the choroidal stroma (Fig. 6A, 6C). This labeling is specific, as its pattern differs from control sections processed with equal concentrations of nonimmune immunoglobulin (Fig. 6B, 6D). ApoA-I immunoreactivity was also detected in plasma retained in retinal vessels (not shown).

**EC Composition of BrM, Lipoproteins, and Ocular Tissues**

The results so far indicate that LLP can be isolated from BrM/Ch. They contain EC, among other lipids, and apos that are detectable in situ within BrM and deposits. Although lending credence to the notion of one or more species of BrM lipoprotein particles, these data do not elucidate the potential source of these lipoproteins. The particles differ from plasma lipoproteins in a key property, behavior in a density gradient, but they resemble plasma lipoproteins, especially LDL, in EC composition. Here, we sought insight by investigating BrM EC composition further, for two reasons. First, hot-stage polarizing microscopy studies have shown birefringence with EC-like crystalline morphology and thermal behavior in BrM, drusen, and sclera. This EC-related birefringence disappears at higher temperatures from BrM and drusen than sclera was attributed to differences in the relative saturation of long-chain fatty acids in EC. Second, the high proportion of cholesteryl linoleate relative to cholesteryl oleate in human connective tissues was an important clue implicating insudation of plasma lipoproteins as a process initiator in both atherosclerosis and xanthoma.

The EC mass in native RPE ranged from 1.9 to 24.2 μg/mg cell protein, with the proportion of TC that is esterified ranging from 0.09 to 0.40 (Table 3). Then, by ESI/MS assay, the EC composition in RPE and partially isolated BrM from fresh tissues (Fig. 7) was similar to that in the isolated BrM/Ch LLP fractions (compare with Fig. 2). That is, cholesteryl linoleate was more than two times more abundant than cholesteryl oleate, and little cholesterol docosahexaenoate was detectable. However, a different picture emerged from analysis of EC composition of BrM, cornea, and sclera of paraformaldehyde-preserved eyes. Cornea and sclera, like LDL, are relatively enriched in cholesteryl linoleate, but preserved BrM, like intracellular droplets of activated macrophages, is enriched in cholesteryl oleate (Fig. 8A). We also compared the EC composition of preserved BrM to RPE and retina of the same eyes (Fig. 8C). All three tissues were enriched in cholesteryl oleate, although the total mass of cholesteryl oleate differed substantially.

**FIGURE 2.** EC composition of BrM/Ch LLPs. After BrM/Ch LLPs were isolated and centrifuged, and lipids were extracted from the pooled density (d) fraction and from Peaks 1 and 2. The concentrations of six cholesteryl esters were assayed by ESI/MS and normalized to cholesteryl oleate (18:1, *). See Table 1 for carbon saturation formulas. Error bar, standard deviations (three aliquots for each sample). (A) BrM/Ch particles (d ≤ 1.24 g/mL, n = 3 donors; Peaks 1 and 2, n = 4 donors). (B) EC composition from LDL, VLDL, and CM of normolipemic human plasma.

**FIGURE 3.** Lipoproteins from BrM/Ch and plasma. Negative stain electron microscopy (A–D, F). (A) Particles in Peak 1 were heterogeneous in size and electron density. Some particles were large and solidly electron lucent (arrow). Most had empty interiors with electron-lucent surfaces (arrowhead). (B) Particles in Peak 2 were mostly large and solidly electron lucent (arrowhead). (C) Plasma LDL. (D) Plasma VLDL. (E) Dietary CM. (F) A coated membrane-bounded body in BrM in situ (arrow) in a thin section transmission electron micrograph. RPE is at the top of the image. Arrowheads: basal lamina. (G) Membrane-bound aggregate containing small electron-lucent particles, Peak 1. Bars: (A, B, E, G) 100 nm; (C, D) 50 nm; (F) 500 nm.
among them (6700 ± 1156, 1190 ± 850, and 180 ± 67 nanomoles/g dry weight, respectively), consistent with the previously reported measurable but low EC mass in the neurosensory retina. Finally, in fellow eyes in which BrM, retina, sclera, and cornea were preserved for 7 days or processed fresh, all ocular regions of both eyes were cholesteryl linoleate–enriched (not shown). In fellow eyes preserved or frozen for 3 months, tissues from only the frozen eye was cholesterol linoleate–enriched (not shown). These results raise the possibility that BrM EC composition is affected by long-term storage differently than are esters of plasma lipoprotein origin that accumulate in sclera and cornea.

**DISCUSSION**

Our main finding was that particles resembling lipoproteins isolated from BrM/Ch did not resemble plasma lipoproteins in

**FIGURE 4.** Western blot analysis of apoB and apoA-I in fractions containing BrM/Ch LLP. After BrM/Ch particle isolation and density ultracentrifugation, Peaks 1 and 2 were subjected to Western blot analysis. (A) Stained with anti-apoB antibody, apoB-100 bands were found in both Peaks 1 and 2. Lane 1: Peak 1 from donor 9; lane 2: Peak 2 from donor 9; lane 3: Peak 1 from donor 10; lane 4: Peak 2 from donor 10; lane 5: human plasma. Lane 5 was developed simultaneously with other lanes and scanned separately due to its higher density. (B) Stained with anti-apoA-I antibody, apoA-I was found in both Peaks 1 and 2. Lane 1: 50 ng apoA-I standard; lane 2: 40 μL of Peak 1 from donor 9; lane 3: 40 μL of Peak 2 from donor 9; lanes 4 to 7: 10 μL each of fractions 6, 7, 9, 12 of Peak 1 from donor 9; lane 8: 10 μL of fraction 17 of Peak 2 from donor 9. (C) ApoA-I expressed in human RPE and retina. Lane 1: 10 ng of plasma apoA-I; lane 2: 50 ng of plasma apoA-I; lane 3: 100 μg of native RPE protein; lane 4: 100 μg of retina protein.

**FIGURE 5.** ApoA-I and LCAT expressed in human retina (lane R), RPE (lane P), ARPE-19 (lane 19), and HepG2 (lane H). Total RNA was isolated from human retina, RPE, ARPE-19, and HepG2 cells, and one-step RT-PCR was performed. Expected RT-PCR products of apoA-I and LCAT were 679 and 470 bp, respectively. Lane M: 100-bp DNA ladder; lengths in bp are indicated.

**FIGURE 6.** ApoA-I immunofluorescence and autofluorescence in cryosections of BrM/Ch and drusen. Sections from the peripheral retina of a 63-year-old woman were probed with polyclonal anti-apoA-I (A, C) or nonimmune immunoglobulin (B, D). Primary antibodies were detected with rhodamine-conjugated secondary antibody. (A) ApoA-I immunoreactivity in BrM (arrows). Arrowheads: autofluorescent RPE. (B) Autofluorescence only in RPE and BM. (C) ApoA-I immunoreactivity in drusen (d), enveloped by autofluorescent RPE (arrowheads). (D) Autofluorescence in RPE and BrM, not in drusen. Bar, 40 μm.
density profile, cholesterol distribution, and morphology. Particles were heterogeneous and distributed into two density peaks, including a large particle in a EC-rich Peak 2 that resembles the 80- to 100-nm particles appearing in situ.14,19 Particles in Peak 1 were more heterogeneous, including some Peak 2-type particles, liposome-like structures, and intact coated membrane-bound bodies.43 Eyes differed substantially in TC yield, probably because of differences in drusen load28,44 or BrM cholesterol content, 14 but the proportions of measured lipids were remarkably consistent across eyes. Although it cannot yet be excluded that some BrM particles are transcytosed from the plasma compartment, it appears that BrM/Ch LLPs differ in important ways from plasma lipoproteins.

An important unanswered question is the number of particle classes present in native BrM. Further, if there are multiple classes, they may arise either independently or by interconversion among them by hydrolysis or lipid transfer, inter alia. Given the abundant Peak 2-type particles seen in situ by conventional and lipid-preserving postfixation methods, interconversion seems likely. However, EM studies have described 70-nm-diameter vesicle-like bodies (now known to represent solid particles) within coated membrane-bound bodies and 70- to 110-nm-diameter vesicle-like bodies elsewhere within BrM.43 A range of particles at different stages of maturation or metabolic and/or degradative modification in BrM may be possible.

Our ultrastructural data suggest some BrM particles are remnant lipoproteins modified in the sub-RPE space by currently undefined processes. Early studies indicated that plasma lipoproteins/H1102270 nm in diameter were unlikely to enter arterial intima because of size-restricted transcytosis at the vascular endothelium.45,46 However, larger remnants of VLDL and CM are now known to enter after the hydrolyzing action of endothelial and lipoprotein lipases that leave UC-enriched surface components intact.9 Among particles isolated from arterial intima,9,24,47,48 heterogeneity in morphology (pitting, clustering, or loss of internal lucency) has been interpreted as evidence for enzymatic, oxidative, or other modifications in the extracellular space. Although no modifying mechanism can be excluded for BrM currently, the loss of internal lucency with retention of external surface (see also Ref. 19) resembles VLDL particles exposed to lipase activity in vitro9 and therefore could represent modification in the extracellular space before

### Table 3. Total Cholesterol and Esterified Cholesterol Mass in Native Human RPE

<table>
<thead>
<tr>
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<td>26.4</td>
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Data are expressed as micrograms per milligram protein.

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**Figure 7.** EC composition in native human RPE and BrM. The concentration of cholesterol esterified to long-chain fatty acids denoted by the key was determined by ESI/MS and normalized to that of cholesterol oleate (18:1, †). See Table 1 for carbon saturation formulas. Native RPE was obtained from four donors >60 years of age with grossly normal maculas. BrM/Ch was isolated from a fresh donor eye. Error bars, standard deviation.

**Figure 8.** EC composition in LDL, monocyte-macrophages, and preserved ocular tissues. EC concentrations in extracts were determined by ESI/MS and normalized to the concentration of cholesteryl oleate (18:1, †). See Table 1 for carbon saturation formulas. Error bars, standard deviations. (A) Cornea, sclera, and BrM (n = 10 eyes), compared to LDL. (B) BrM (n = 4 eyes) and cholesterol-loaded monocyte-macrophages. (C) BrM, RPE, and neurosensory retina (n = 6 eyes).
the donor’s death (e.g., hydrolysis by lipases resident in the choroid). It is also possible that some Peak 1 heterogeneity represents vesicles released from cellular membranes during homogenization and sonication inherent in sample preparation.

With regard to an intraocular atherogenic apoB-containing lipoprotein, our data do not fully resolve the crucial question of apo identity. Western blot analysis convincingly demonstrated apoB-100 as well as a lower-molecular-weight band consistent with either apoB-48 or an apoB-100 degradation product. Such bands frequently appear in apoB Western blots, because apoB is readily proteolyzed at predictable sites during isolation, as an intracellular regulatory mechanism, and as an indicator of translocation arrest in the absence of microsomal triglyceride transfer protein. Our data also do not resolve the crucial question of whether BrM particles are TG-rich, as predicted by their size and the chromatographic evidence that the TG/EC molar ratio in intact BrM/Ch is 1.77. Perhaps a more sensitive technique for TG detection (e.g., Ref. 53) would provide a different answer. Alternatively, low TG levels may reflect the results of lipolytic enzyme activity in the sub-RPE space.

The conclusions of prior compositional studies on the source of BrM lipids were based on lipids not exclusively localized to BrM (i.e., PL25 or UC14), which could derive from membranes of incompletely removed choroidal cells. Despite the expectation that fatty acyl residues in BrM EC should reflect composition of potential sources, such sources are not conclusively identified by our study. An attractive hypothesis is that these residues represent direct or indirect degradative products emanating from outer segment phagocytosis. However, because BrM EC has little cholesteryl docosahexaenoate or cholesteryl stearate, the two most abundant fatty acids in outer segment PL, it does not resemble outer segment PL, in either preserved or fresh eyes (Fig. 9). These results are consistent with an intermediary mechanism with specific substrate preferences that repackages fatty acids for neutral lipid secretion. Leading candidate mechanisms are diacylglycerol acyl transferase and acyl cholesterol acyl transferase, which catalyze the final committed steps in TG and EC synthesis, respectively, and stearoyl-CoA-desaturase, which catalyzes the desaturation of stearic acid to oleic acid. Gene expression of LCAT, which produces a cholesteryl ester, is expressed in the brain, where it may esterify cholesterol on UC-rich lipoprotein particles secreted by astrocytes. It will be interesting to determine whether RPE LCAT gene expression signifies a capacity to synthesize linoleate-enriched cholesteryl esters in the sub-RPE space.

The finding that BrM EC is cholesteryl oleate enriched in preserved eyes and cholesteryl linoleate enriched in fresh eyes and isolated BrM particles was unexpected, yet consistent with the work of Haimovici et al., applying a physicochemical technique to cryosections of paraformaldehyde-preserved normal aged eyes. The basis of this paradoxical finding is unknown, because all preserved eye parts were stored together, but it underscores that BrM and retinal ECs differ from that in connective tissue. Selective modification (e.g., oxidation of cholesteryl linoleate) during storage is a possible explanation for this discordant finding in two laboratories.

Detection of apoA-I in RPE and neurosensory retina, extending proteomics results, expands the retinal apo gene expression repertoire. Weak bands detected at 56 and 84 kDa with an affinity-purified antibody are most likely the dimeric and trimeric forms of apoA-I, respectively, as apoA-I self-associates in solution. With these data, drusen are now known to contain apoB, apoE, and apoA-I. With regard to RPE, it remains to be determined whether apoA-I protein is secreted on a small HDL-like particle, or more intriguingly, on a novel, large CM-like particle. This notion is appealing, because RPE lipoprotein release after outer segment phagocytosis could be conceptualized as a postprandial event, similar to CM release. Whether all three apos occupy the same or different particles in BrM can be answered indirectly, by immunogold electron microscopy of particles in situ, or directly, by isolating and characterizing the full range of particles from appropriately lipid-loaded RPE cells in vitro.

In summary, LLP in fractions containing apoB, apoA-I, and cholesterol can be isolated from normal human BrM/Ch. Cholesteryl ester composition and interocular expression of genes encoding apop2 augment circumstantial evidence that particles are formed within the eye. There, they may contribute to age- and ARM-related drusen and basal linear deposits in a process analogous to atherosclerosis initiation in arterial intima by accumulation of plasma apoB-containing lipoproteins.

Acknowledgments

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References


