

# Local Cellular Sources of Apolipoprotein E in the Human Retina and Retinal Pigmented Epithelium: Implications for the Process of Drusen Formation

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- **PURPOSE:** The inheritance of specific apolipoprotein E alleles has been linked to atherosclerosis, Alzheimer disease, and, most recently, to the incidence of age-related macular degeneration. Apolipoprotein E is a common component of the extracellular plaques and deposits characteristic of these disorders, including drusen, which are a hallmark of age-related macular degeneration. Accordingly, we assessed the potential biosynthetic contribution of local ocular cell types to the apolipoprotein E found in drusen.
- **METHODS:** We measured apolipoprotein E mRNA levels in human donor tissues using a quantitative assay of apolipoprotein E transcription, and we localized apolipoprotein E protein to specific cell types and compartments in the neural retina, retinal pigmented epithelium, and choroid using laser scanning confocal immunofluorescence microscopy.
- **RESULTS:** Apolipoprotein E immunoreactivity is associated with photoreceptor outer segments, the retinal ganglion cell layer, the retinal pigmented epithelium basal

cytoplasm and basal lamina, and with both collagenous layers of Bruch membrane. Apolipoprotein E appears to be a ubiquitous component of drusen, irrespective of clinical phenotype. It also accumulates in the cytoplasm of a subpopulation of retinal pigmented epithelial cells, many of which overlie or flank drusen. Mean levels of apolipoprotein E mRNA in the adult human retina are 45% and 150% of the levels measured in liver and adult brain, the two most abundant biosynthetic sources of apolipoprotein E. Apolipoprotein E mRNA levels are highest in the inner retina, and lowest in the outer retina where photoreceptors predominate. Significant levels of apolipoprotein E mRNA are also present in the retinal pigmented epithelium/choroid complex and in cultured human retinal pigmented epithelial cells.

- **CONCLUSIONS:** Apolipoprotein E protein is strategically located at the same anatomic locus where drusen are situated, and the retinal pigmented epithelium is the most likely local biosynthetic source of apolipoprotein E at that location. Age-related alteration of lipoprotein biosynthesis and/or processing at the level of the retinal pigmented epithelium and/or Bruch membrane may be a significant contributing factor in drusen formation and age-related macular degeneration pathogenesis. (Am J Ophthalmol 2001;131:767-781. © 2001 by Elsevier Science Inc. All rights reserved.)

Accepted for publication Dec 11, 2000.

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This work was supported by research Grants EY-11521 (Dr Anderson), EY-11515 (Dr Hageman), and EY-11527 (Dr Johnson) from the National Eye Institute, National Institutes of Health, Bethesda, Maryland; a National Eye Institute Core Grant for Vision Research (EY-01931) and an unrestricted grant from Research to Prevent Blindness awarded to the Department of Ophthalmology, Medical College of Wisconsin, Milwaukee, Wisconsin.

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**A** POLIPOPROTEIN E IS A MULTIFUNCTIONAL, LIPOPHILIC glycoprotein that plays a major role in the transport of lipids from cellular sites of synthesis or uptake to sites of utilization or clearance.<sup>1</sup> In plasma, apolipoprotein E is a constituent of very low density lipoprotein, a subclass of high density lipoprotein, and chylomicrons. As one of its major functions, apolipoprotein E facilitates the uptake of cholesterol, triglycerides, and other lipids by hepatic and other cells. In this capacity,

it serves as a ligand for several members of the low density lipoprotein receptor family.<sup>2</sup> In the brain, apolipoprotein E appears to mediate the repair and recovery of injured and diseased neurons, with specific isoforms being more or less effective in that regard.<sup>3</sup>

The inheritance of specific apolipoprotein E alleles in the human population has been linked to the age of onset in atherosclerosis<sup>4</sup> and Alzheimer disease.<sup>5,6</sup> The presence of the  $\epsilon 2$  allele is associated with hyperlipidemia and premature atherosclerosis, whereas the  $\epsilon 4$  allele correlates with an increased risk of coronary artery disease, and the common late-onset familial and sporadic forms of Alzheimer disease. Apolipoprotein E polymorphisms may also be correlated with the incidence of age-related macular degeneration, the leading cause of irreversible blindness in many countries.<sup>7</sup> In age-related macular degeneration, however, the  $\epsilon 4$  allele appears to confer protection, whereas the  $\epsilon 2$  allele is apparently associated with a moderately increased incidence of the disease.<sup>8,9</sup>

One of the hallmarks of age-related macular degeneration is the accumulation of deposits along Bruch membrane, a stratified extracellular complex that separates the retinal pigmented epithelium and the neural retina from the adjacent choroid. The most prevalent type of deposit, drusen, is located at the boundary between the basal lamina of the retinal pigmented epithelium and the inner collagenous layer of Bruch membrane. Although occasional drusen can be present in the eyes of relatively young individuals, their size and numbers tend to increase with advancing age. Those with high numbers of drusen, large drusen, or confluent drusen, especially within the macula, incur a substantially increased risk of developing age-related macular degeneration. As such, determining the molecular composition of drusen and identifying the cellular sources of those molecules could provide significant new insights into the process of drusen biogenesis and the pathogenesis of age-related macular degeneration.

Although drusen were first described almost 150 years ago, information bearing on their origin and molecular composition remains sparse. They have been reported to contain neutral lipids,<sup>10,11</sup> free and esterified cholesterol,<sup>12</sup> various glycoconjugates,<sup>13,14</sup> as well as several basement membrane components.<sup>15,16</sup> Most recently, a number of additional proteins have been identified that are components of both hard and soft drusen,<sup>9,17-19</sup> the two main, clinically defined phenotypes.<sup>20</sup> Among these are a number of immune-related and inflammatory-related proteins, including vitronectin, amyloid P component, and C-reactive protein; several terminal complement components, including the membrane attack complex; factor X, prothrombin, and a major histocompatibility complex class II component (HLA-DR).<sup>14,21-23</sup> Also present are immunoglobulins that are apparently limited to the gamma subclass.<sup>21,23</sup> Collectively, these findings strongly suggest that activation of the terminal pathway of the complement cascade is an integral part of the process of drusen

formation, and they help to explain why a significant number of molecules that can activate or modulate the terminal pathway are associated with drusen.

Interestingly, a number of drusen-associated molecules including apolipoprotein E, vitronectin, amyloid P component, and C-reactive protein are also prominent molecular components of the age-related plaques and extracellular deposits associated with atherosclerosis, amyloidoses, dermal elastosis, dense deposit disease, and neurodegenerative brain disease.<sup>23</sup> Although similar in composition, drusen are apparently not an ocular manifestation of any of these other diseases, but common pathogenic pathways, including complement activation, may be involved in their formation.

Many drusen-associated molecules are known to be synthesized primarily in the liver before being released into the circulation.<sup>1,24-26</sup> Accordingly, their extravasation from the choroidal capillaries, and their subsequent aggregation along Bruch membrane, constitute one potential deposition pathway. However, local cell types in the neural retina, retinal pigmented epithelium, and/or choroid that lie in close proximity to Bruch membrane also have the capacity to synthesize a number of these molecules.<sup>22,23,26,27</sup>

In this study, we present new evidence regarding the potential contribution of local cell types to the apolipoprotein E in drusen. Specifically, we identify the retinal pigmented epithelium as an abundant source of apolipoprotein E transcripts. We show that apolipoprotein E protein is associated with the retinal pigmented epithelium and with Bruch membrane, and we show that it accumulates in the cytoplasm of a subpopulation of retinal pigmented epithelial cells, many of which flank or overlie drusen. These results reinforce the apparent linkage between the retinal pigmented epithelium and the process of drusen formation,<sup>21</sup> and lay the foundation for further studies aimed at determining if age-related or inherited alterations in retinal pigmented epithelial lipoprotein processing or clearance contribute to age-related macular degeneration pathogenesis.

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## METHODS

A TOTAL OF 35 HUMAN DONOR EYES WAS OBTAINED FROM either the University of Iowa Center for Macular Degeneration (Iowa City, Iowa), Mid America Transplant Services (St. Louis, Missouri), Doheny Eye and Tissue Transplant Bank of the Central Coast (Goleta, California), the Eye Institute at the Medical College of Wisconsin (Milwaukee, Wisconsin), or the Lions Eye Bank of Oregon (Portland, Oregon). Recent medical histories were obtained from all donors at the time of death, as were ophthalmic histories when available. Tissue specimens from 18 donor eyes were processed for immunohistochemical analysis. Donor ages ranged from 15 to 93 years of age,

and postmortem times to fixation ranged from 1 to 15 hours, with most being less than 8 hours. For quantitative analyses of mRNA levels, total RNA was isolated from the neural retina and retinal pigment epithelium/choroid of 17 donor eyes within 2 to 6 hours of death. The age range of these donors was 34 to 98 years.

After removal of the anterior segments, the posterior poles were examined under a stereo microscope for evidence of drusen, geographic atrophy, choroidal neovascularization, or other signs of retinal pathology, and then photographed. Posterior poles were dissected into quadrants. In some cases, a corneal trephine was used to obtain 6-mm diameter punches from the macula, and other retinal locations. At that point specimens were either fixed for microscopy, as described below, or processed for mRNA quantitation. For mRNA quantitation, the neural retinas were carefully peeled away from the retinal pigmented epithelium/choroid/sclera using fine forceps with the aid of a stereo microscope. Next, the retinal pigmented epithelium and adherent choroid was dissected, and the sclera was discarded. Retinal and retinal pigmented epithelium/choroid tissue specimens were transferred immediately to individual cryovials, snap frozen in liquid nitrogen, and stored at  $-70^{\circ}\text{C}$ .

The posterior poles were submerged for several hours in cold Dulbecco's modified Eagle's medium (DMEM) tissue culture medium supplemented with 10% sucrose. Subsequently, the neural retina was dissected from each eyecup leaving the choroid and retinal pigmented epithelium behind. The retina was placed in a Petri dish, and submerged in culture medium. A trephine was used to punch a 6-mm patch of retina centered on the fovea. In some eyes, a cleavage occurs at the level of the outer limiting membrane. This separation occurred readily in some retinas, but not in others, and had no apparent relationship to the age of the donor or to the postmortem interval. In several eyes where this fortuitous separation was visible using a dissecting microscope, small slabs of the distal retina containing the photoreceptor inner/outer segments were teased away with the aid of small forceps. The remainder of the retina was inspected carefully under the dissecting microscope to make sure the entire layer of inner/outer segments had been removed. This proximal retina was then transferred to a bath of fresh culture medium and rinsed several times in fresh medium. Specimens that consisted of either the distal or proximal portions of the retina were then harvested and transferred to individual microfuge tubes. The tubes were centrifuged briefly to pellet the tissues, and the supernatant was removed by pipette without further agitation. Finally, the tubes were placed in a dry ice/ethanol bath and then stored at  $-80^{\circ}\text{C}$ .

Human ocular tissues were procured from Advanced Bioscience Resources (Alameda, California). HepG2 cells were obtained from American Type Culture Collection (Manassas, Virginia). The harvesting and isolation condi-

tions for the fetal human retinal pigmented epithelial cell primary cultures are described in Pfeffer.<sup>28</sup> Retinal pigmented epithelial and HepG2 cells were grown and maintained in Dulbecco modified Eagle medium (Mediatech, Herndon, Virginia) supplemented with 10% fetal calf serum (Fetal Clone II; Hyclone, Logan, Utah), as described in Ozaki and associates.<sup>26</sup>

All tissues were processed for immunohistochemistry using the procedures reported recently elsewhere.<sup>21</sup> The methods and procedures used for laser scanning confocal immunofluorescence microscopy, as described initially by Matsumoto and Hale,<sup>29</sup> are described in detail in Anderson and associates.<sup>30</sup> For the dual confocal immunofluorescence labeling, 3-mm to 4-mm diameter tissue specimens were incubated overnight in a buffered solution containing the two primary antibodies. The two secondary antibody fluorochrome conjugates (donkey antirabbit and goat antimouse immunoglobulin gamma-Cy2 or Cy3 (Jackson ImmunoResearch Laboratories, West Grove, Pennsylvania) were also combined in the subsequent detection step. For apolipoprotein E/immunoglobulin gamma dual labeling, the sections were incubated initially with a combined solution containing rabbit antihuman apolipoprotein E polyclonal antibody (Dako Corp, Carpinteria, California) and a donkey anti-human immunoglobulin gamma-Cy3 conjugate (Jackson); that was followed by incubation in a second solution containing donkey antirabbit-Cy2 conjugate (Jackson). In some cases, a pseudo-dual labeling procedure was used to distinguish retinal pigmented epithelium autofluorescence attributable to lipofuscin (600 nm fluorescence emission peak)<sup>31,32</sup> from immunoreactivity. Using this procedure, gain and laser power levels were adjusted to visualize lipofuscin granules on the Cy3 (red) channel, and the immunofluorescence signal was captured and displayed on the Cy2 (green) channel. By capturing the images sequentially and then merging them, lipofuscin autofluorescence and immunofluorescence were visualized simultaneously.

All primary antibodies were used at concentrations ranging from 5 to 20  $\mu\text{g/ml}$ . Negative controls in which one (or both) primary antibodies were omitted were included in each run. In selected cases, an irrelevant primary antibody at the same concentration was substituted for the appropriate primary antibody(ies). All positive results were verified in at least two and, in most cases, three serial runs.

Primary antibodies included a polyclonal antibody raised in rabbit against human recombinant apolipoprotein E (stock concentration 5.9 g/l; Dako Corporation, Carpinteria, California); a vitronectin polyclonal antibody adsorbed against human plasma proteins<sup>33</sup> (Life Technologies, Rockville, Maryland); vitronectin monoclonal antibody 8E6<sup>33</sup> (Life Technologies); monoclonal antibody 16A7<sup>30,34</sup> (supplied as a gift by Professor Klaus Preissner); and a monoclonal antibody to platelet-derived endothelial cell adhesion molecule-1 clone JC/70A (Dako Corpora-

tion). The nucleic acid-binding cyanine dye, TO-PRO-3 (Molecular Probes, Eugene, Oregon), was used as a probe for single-stranded DNA, double-stranded DNA, and RNA. A small volume of the dye was added directly to the buffer solution containing the primary antibody and applied to sections at a final dilution of 1:3000 from stock concentration.

The apolipoprotein E antibody was characterized on Western blots of apolipoprotein E purified from human plasma. Five hundred ng of purified apolipoprotein E (Calbiochem; La Jolla, California) was loaded and run on 12.5% polyacrylamide gels under reducing conditions, blotted to nitrocellulose membrane for 1 hour using borate blot buffer (0.45  $\mu$ m), incubated for 1 hour in buffer containing the apolipoprotein E antibody at a 1:1000 dilution of the stock solution, probed for 1 hour in buffer containing an antirabbit immunoglobulin gamma-alkaline phosphatase conjugate (1:30,000 dilution of stock solution; Sigma, St. Louis, Missouri), and then detected using a Vectastain alkaline phosphatase substrate kit (Vector Laboratories, San Mateo, California). Under these conditions, the antibody recognized a 35 kDa triplet that approximates the molecular mass of human apolipoprotein E, and no other gel components.

A Qiagen RNeasy kit (Valencia, California) was used to extract total RNA from the following tissue sources: neural retina; retinal pigmented epithelium/choroid complex; primary cultures of human fetal retinal pigmented epithelial cells (C-retinal pigmented epithelium), HepG2 cells,<sup>35</sup> and normal adult human liver (obtained within 2 hours of biopsy as a gift from Dr Harvey Solomon, Department of Surgery, St. Louis University School of Medicine). In addition, total RNAs from the following organs were purchased from either CLONTECH Laboratories, Inc (Palo Alto, California) or Origene, Inc (Rockville, Maryland): whole normal brain pooled from 24 spontaneously aborted fetuses (lot number 6120259; Clontech); whole normal brain pooled from two females aged 16 and 36 years (lot number 7080717); and whole normal liver pooled from two donors aged 15 and 35 years (lot number 7040868).

After DNase treatment, cDNAs were synthesized using random hexamer primers in the presence or absence of Superscript II reverse transcriptase (Gibco BRL, Gaithersburg, Maryland) according to the manufacturer's instructions. After reverse transcriptase, cDNAs were amplified using the polymerase chain reaction and then quantified in real-time using automated sequence detection instrumentation (Prism 7700 Sequence Detector; PE Applied Biosystems, Foster City, California) that incorporates a dual-label fluorogenic detection system (Taqman)<sup>36</sup> based on the 5' nuclease assay.<sup>37</sup> Quantitative reverse transcriptase-polymerase chain reaction was performed as described previously<sup>26</sup> with cDNA as template using the appropriate gene-specific primer (300 nM) and probe (200 nM) combination. An 18S ribosomal RNA (18S rRNA) primer (50 nM) and probe (50 nM) was employed as an endogenous control to correct for sample-to-

sample variation in initial RNA concentration, RNA integrity, and efficiency of the reverse transcriptase reaction. Primers and probes were designed with the assistance of the Prism 7700 sequence detection software (Primer Express, PE ABI, Foster City, California). The following primer/probe sequences were used:

18S rRNA:

Primers F: 5'-GCCGCTAGAGGTGAAATTCTT-3'

B: 5'-CATTCTTGGCAAATGCTTTTCG-3'

Probe: 5'FAM-ACCGGCGCAAGACGGACCAGA-3'TAMRA

Apolipoprotein E:

Primers F: 5'-CTGGCACTGGGTCGCTTTT-3'

B: 5'-AGTTGTTCCCTCCAGTTCGGATTT-3'

Probe: 5'FAM-AGGGCGCTGATGGACGAGACCA-3'TAMRA

Amplification reactions were performed in MicroAmp Optical tubes (PE ABI) containing a total volume of 25  $\mu$ l of the following reagents: 1  $\times$  Taqman polymerase chain reaction buffer containing a reference dye (ROX), 5.5 mM MgCl<sub>2</sub>, AmpliTaq Gold DNA polymerase (0.025 U/ $\mu$ l), AmpErase uracil N-glycosylase (UNG, 0.01 U/ $\mu$ l), 200  $\mu$ M each dATP, dGTP, dCTP, and 400  $\mu$ M dUTP (PE ABI). Polymerase chain reaction amplification was carried out using the following temperature profile: 2 minutes at 50C and 10 minutes at 95C; followed by 40 cycles at 95C (15 seconds) and 60C (60 seconds).

Data were analyzed using the relative standard curve method as described in the PE Applied Biosystems User Bulletin number 2 (1997). Standard curves of apolipoprotein E and 18S rRNA were generated using cDNAs synthesized from 1:5 serial dilutions of total liver RNA. For each experimental sample, the amounts of apolipoprotein E mRNA and 18S rRNA were extrapolated from the standard curves. The normalized values were calculated by dividing the mean apolipoprotein E quantity by the mean 18S rRNA value for each sample. The normalized values for the target samples were expressed and plotted as a fraction of the reference standard (that is, liver or brain), which was arbitrarily set at 1.0. Statistical analyses of the data were identical to that described in Ozaki and associates.<sup>26</sup>

## RESULTS

IN THE OUTER NEURAL RETINA, APOLIPOPROTEIN E IMMUNOREACTIVITY IS ASSOCIATED PRINCIPALLY WITH ROD AND CONE



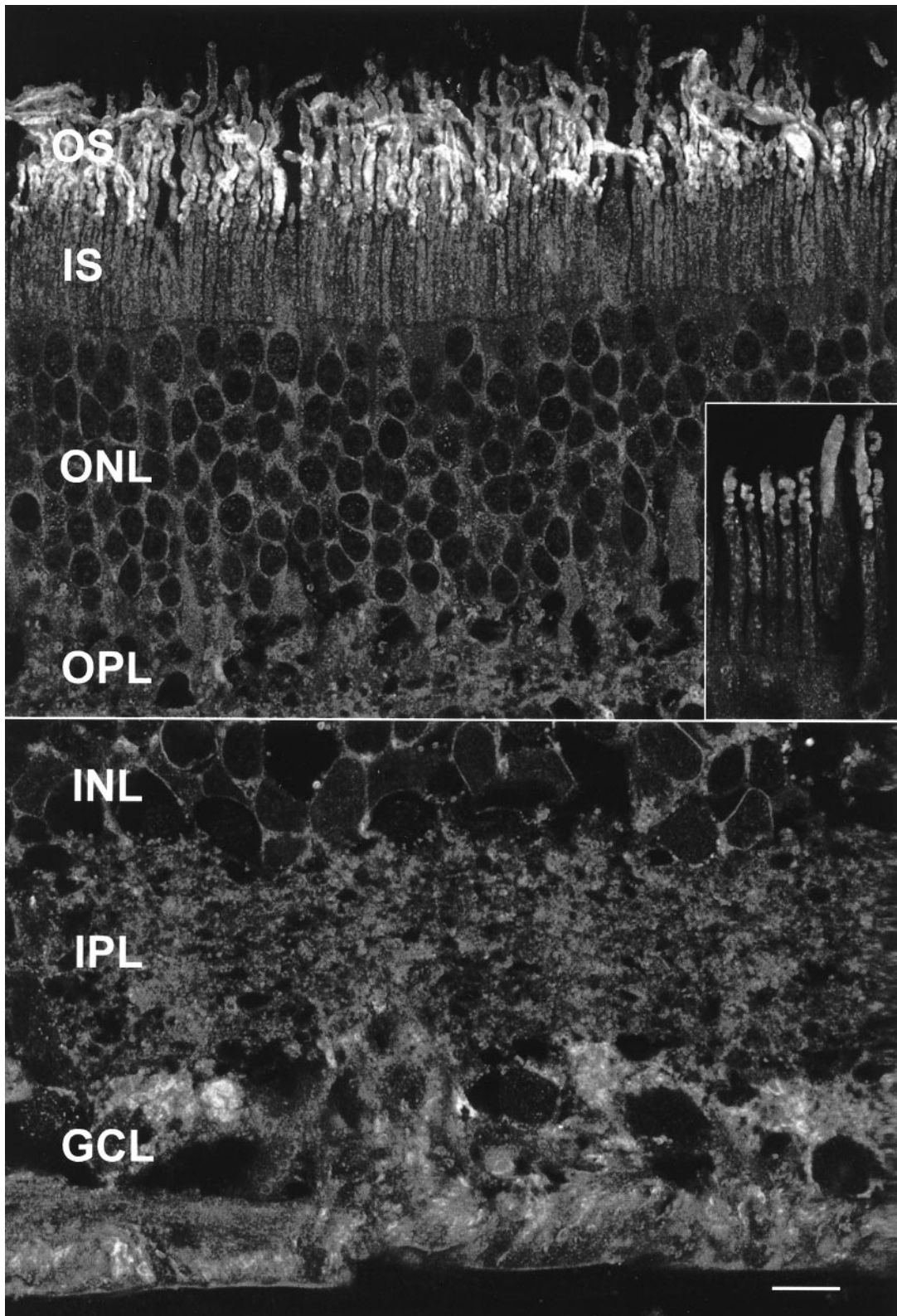


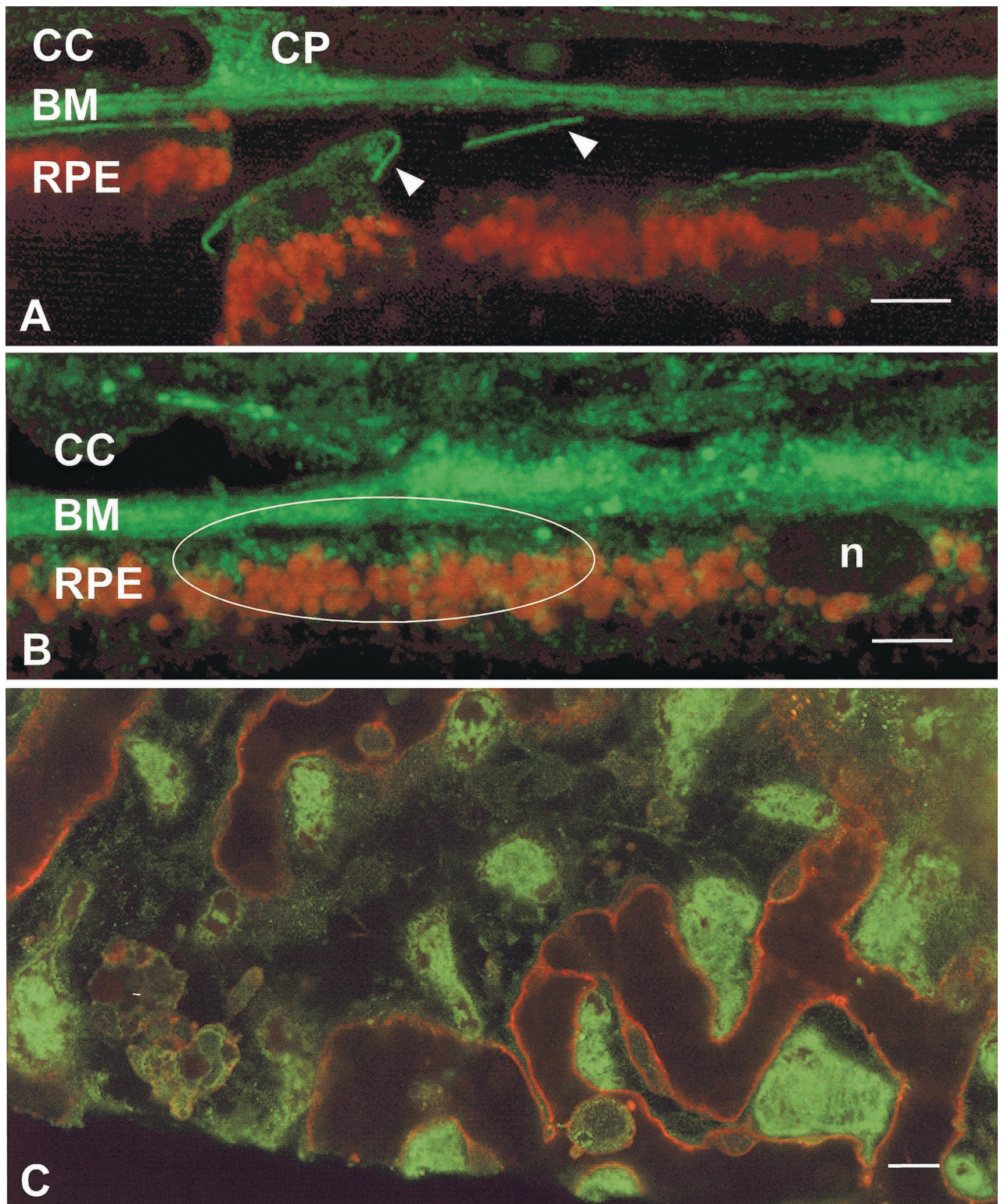
FIGURE 1. Confocal immunofluorescence images of apolipoprotein E immunoreactivity in the adult human retina. (Top) In the outer retina, apolipoprotein E immunoreactivity is associated primarily with rod and cone outer segments (OS; inset). Photoreceptor inner segments (IS) show a small amount of punctate labeling; neither the outer nuclear layer (ONL) nor the outer plexiform layer (OPL) shows any appreciable labeling. (Bottom) In the inner retina, there is some diffuse labeling within the ganglion cell layer (GCL). No labeling of the inner nuclear (INL) or inner plexiform layers (IPL) is apparent in single optical sections. Scale bar = 5  $\mu\text{m}$ .



photoreceptor outer segments, with weak punctate labeling also present over inner segments (Figure 1). In the inner neural retina, labeling is relatively sparse in single optical sections, except for some diffuse labeling within the ganglion cell layer (Figure 1). In projection series, perinuclear labeling of some cell bodies in both the inner nuclear

layer and ganglion cell layer is apparent. In addition, the tunica media of large retinal vessels is labeled, but there is no corresponding labeling of the intima.

Several of the strata of Bruch membrane react with apolipoprotein E antiserum, including a layer adherent to the basal retinal pigmented epithelial surface that most





likely corresponds to its basal lamina, and two more distal layers that represent the inner and outer collagenous layers respectively (Figure 2, A). The intervening elastic layer is unlabeled. In projection series, a population of small apolipoprotein E-rich granules is present in the basal cytoplasm or basal retinal pigmented epithelium surface (Figure 2, B). Some labeling is also scattered throughout the choroid, but it is concentrated primarily in fibril-rich regions between the fenestrated capillaries of the choriocapillaris (capillary pillars; Figure 2, C).

At the basolateral borders of hard drusen, the trilaminar pattern of apolipoprotein E immunoreactivity in Bruch membrane is interrupted (Figure 3, A and B). The separation between the presumptive retinal pigmented epithelium basal lamina and the inner collagenous layers is no longer evident, and these two formerly apposing layers become indistinguishable from the drusen hemisphere (Figure 3, B). Little or no apolipoprotein E immunoreactivity is evident in the smaller spherical and elliptical structures that are sometimes embedded in hard drusen (Figure 3, C and D). Double-labeling experiments show that the distribution of apolipoprotein E immunoreactivity in Bruch membrane, in smaller hard drusen, and in the capillary pillars is similar to that of immunoglobulin gamma (Figure 3, D). However, the immunoglobulin gamma distribution in the choriocapillaris and in the choroidal stroma is more widespread.

We performed additional double-labeling experiments to examine the extent to which the apolipoprotein E localized in Bruch membrane, drusen, and the choriocapillaris co-distributes with vitronectin, another prominent drusen constituent.<sup>38</sup> In Bruch membrane, the distributions of vitronectin and apolipoprotein E overlap, but they are not identical. Like apolipoprotein E, vitronectin immunoreactivity is prevalent in the inner collagenous zone, but in contrast to apolipoprotein E, the presumptive retinal pigmented epithelium basal lamina is not immunoreactive for vitronectin (Figure 3, E and F). Projection series show that focal accumulations of vitronectin and apolipoprotein E immunoreactivity are frequently present at the basolateral margins of both large and small hard

drusen (Figure 3, E) and the subadjacent capillaries (Figure 3, G). In some larger hard drusen the apolipoprotein E/vitronectin immunoreactivity tends to be concentrated on the outer surface or cortex of the druse (Figure 3, E), whereas in some others it may be excluded completely. It is unclear whether the interiors of these larger drusen are actual exclusion zones that could, for example, be calcified; or, whether absence of labeling is a manifestation of incomplete antibody penetration during processing.

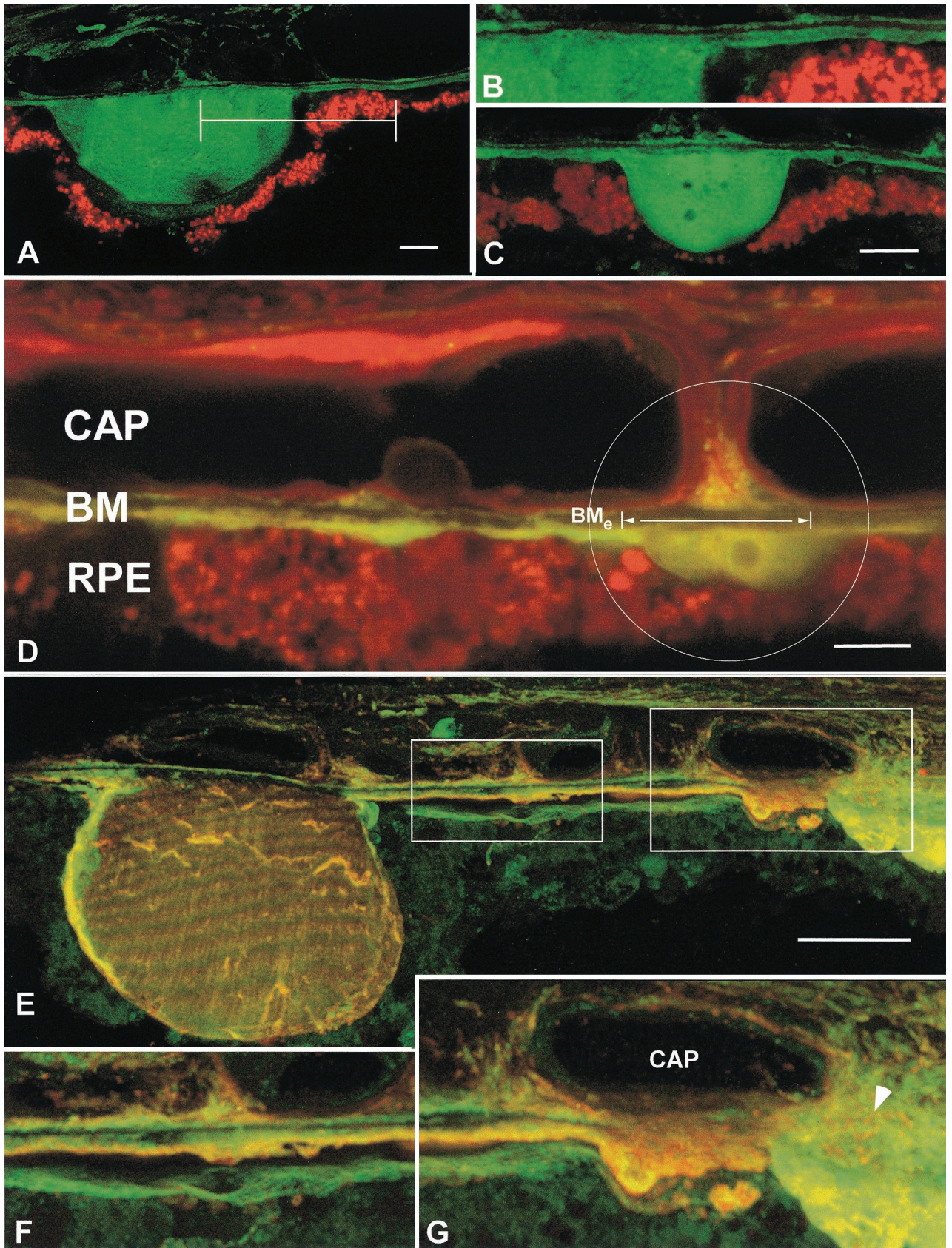
In those donor eyes where numerous drusen are present, intense apolipoprotein E cytoplasmic immunoreactivity is seen in a small subpopulation of retinal pigmented epithelial cells. These cells are most frequently observed on the flanks of hard drusen (Figure 4, A) and immediately overlying drusen (Figure 4, B). However, such cells are also interspersed among nonreactive retinal pigmented epithelial cells in regions of the monolayer where drusen are absent (Figure 5). By confocal microscopy, pseudo-double-label imaging reveals that the apolipoprotein E cytoplasmic immunoreactivity does not co-localize with lipofuscin granules (Figure 5).

Hard drusen sometimes contain a spherical core located at or near the base of the druse adjacent to Bruch membrane (Figure 6, A to E). Discrete foci of vitronectin and/or apolipoprotein E immunoreactivity can also be visualized within even the smallest drusen (Figure 6, A). In some cases, these foci appear as small spheres (less than 5  $\mu\text{m}$  in diameter) that are immunoreactive for both apolipoprotein E and vitronectin (Figure 6, A). In other instances, they consist of a nonimmunoreactive core surrounded by a concentric ring of apolipoprotein E and/or vitronectin (Figure 6, A to D). Although vitronectin immunoreactivity is usually absent from such cores, a punctate pattern of apolipoprotein E immunoreactivity can sometimes be identified within those that are larger and more well defined (Figure 6, D). Surrounding the core is a "halo" of apolipoprotein E and vitronectin immunoreactivity that tapers off sharply at increasing distances from the core's center. Serial optical sectioning shows that at least some of these cores may be in continuity with the elastic layer of Bruch membrane (Figure 6, D). Although

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**FIGURE 2** (facing page). Confocal immunofluorescence images of apolipoprotein E immunoreactivity in the adult human retinal pigmented epithelium (RPE)/choroid. (A) Apolipoprotein E immunolabeling of the retinal pigmented epithelium and Bruch membrane (BM; Cy2 [green] channel). Bruch membrane exhibits a trilaminar anti-apolipoprotein E labeling pattern. In this field, several retinal pigmented epithelial cells have detached artifactually from Bruch membrane revealing a thin band of immunoreactivity that corresponds to the retinal pigmented epithelium basal lamina (arrowheads), the most proximal layer of Bruch membrane. Apolipoprotein E labeling is also associated with two more distal layers that correspond to the inner and outer collagenous layers of Bruch membrane. Also note the intense labeling of the capillary pillars (CP), the extracellular region between the choroidal capillaries (CC). The gain on the Cy3 channel (shown in red) has been adjusted to highlight the autofluorescent lipofuscin granules in the retinal pigmented epithelium cytoplasm. Scale bar = 5  $\mu\text{m}$  (B) confocal projection series of ten 0.8- $\mu\text{m}$  serial optical sections. A population of apolipoprotein E-rich granules is present on the basal retinal pigmented epithelial surface and/or cytoplasm that does not co-localize with the autofluorescent lipofuscin granules (ellipse). n = retinal pigmented epithelium nucleus. Scale bar = 5  $\mu\text{m}$  (C) En face image of the choriocapillaris and the intercapillary zone. A monoclonal antibody to PECAM-1 (JC/70A) is used to visualize the walls of the anastomosing capillary network (Cy3 channel [red]). Foci of anti-apolipoprotein E labeling are concentrated in the capillary pillars (Cy2 channel [green]). Scale bar = 10  $\mu\text{m}$ .







some cores have the dimensions appropriate for a cell nucleus (Figure 7, D), no evidence of nucleic acid is detected using a fluorescent probe (TOPRO-3) with high binding affinity for both single and double-strand DNA, as well as RNA (Figure 6, E).

Having established that the mRNAs for apolipoprotein E and vitronectin, but not amyloid P component, are present in the normal adult retina, retinal pigmented epithelium/choroid, and in cultured human retinal pigmented epithelial cells,<sup>22,23,30</sup> we measured the relative amounts of apolipoprotein E mRNA in these tissues/cells in relation to the levels present in adult liver and brain where apolipoprotein E is thought to be expressed at the highest levels (Figure 7). The quantitative reverse transcriptase–polymerase chain reaction data show that the mean level of apolipoprotein E mRNA in the adult brain is approximately 30% of that measured in the liver reference standard, thus confirming previous studies indicating that the brain is a prominent secondary source of apolipoprotein E biosynthesis.<sup>39</sup> In the neural retina, the mean apolipoprotein E mRNA level obtained from 15 adult human donors (Table 1) is about 150% of the value in adult brain, and approximately 45% of the liver standard, a level that approximates the vitronectin mRNA retina/liver ratio.<sup>26</sup> Apolipoprotein E mRNA levels in the retinal pigmented epithelium/choroid are significantly lower relative to liver (14%), and less than one third of the mean value in the retina. In the cultured cells, mean apolipoprotein E mRNA levels in retinal pigmented epithelial cells are 33% of the liver standard value; in HepG2 cells they represent approximately 25% of the standard (Figure 7). Thus, apolipoprotein E mRNA levels in con-

fluent cultures of human fetal retinal pigmented epithelial cells are at least equal to, and perhaps somewhat greater than, those present in the liver-derived HepG2 cell line. Similar comparisons between retinal pigmented epithelium and HepG2 cells show that their vitronectin mRNA levels are also approximately equivalent.<sup>26</sup>

Based on their apolipoprotein E immunoreactivity profile, the photoreceptors and/or retinal pigmented epithelial cells seem to be the most likely candidates involved in local apolipoprotein E biosynthesis. In order to further clarify this issue, we compared apolipoprotein E mRNA levels in tissue fractions derived from photoreceptor outer/inner segments, inner retina, or retinal pigmented epithelium/choroid, respectively (see Methods). Virtually no apolipoprotein E mRNA is detected in the photoreceptor cell fraction, although substantial amounts are found in both the inner retina and retinal pigmented epithelium/choroid (Figure 8).

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## DISCUSSION

RECENTLY WE REPORTED THAT VITRONECTIN, A TERMINAL complement inhibitor and an abundant plasma glycoprotein synthesized primarily in the liver, is a ubiquitous component of both hard and soft drusen.<sup>14,22</sup> In a subsequent study, we reported that drusen contain a number of additional proteins, some of which are either complement components or modulators of the terminal complement pathway.<sup>21,23</sup> These results raised the possibility that local ocular cell types in the retina, retinal pigmented epithelium, and/or choroid could contribute to the accumulation

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FIGURE 3 (facing page). Dual-label confocal immunofluorescence images of apolipoprotein E, immunoglobulin gamma, and vitronectin in Bruch membrane (BM) and in drusen. (A to C) Apolipoprotein E distribution in hard drusen (Cy2 [green] channel). Lipofuscin autofluorescence (Cy3 [red] channel). (A) In many smaller hard drusen, apolipoprotein E is distributed homogeneously and is in apparent continuity with the apolipoprotein E in Bruch membrane. Scale bar = 10  $\mu$ m. (B) Area enclosed by brackets in A at threefold higher magnification. The apolipoprotein E immunoreactivity in drusen is continuous with the proximal layers of Bruch membrane. (C) Apolipoprotein E immunoreactivity is absent in the small spherical and elliptical granules that are often present in hard drusen. Scale bar = 10  $\mu$ m. (D) Dual-label confocal image showing the overlapping distributions of apolipoprotein E (Cy2 [green] channel) and immunoglobulin gamma (Cy3 [red] channel) in drusen, Bruch membrane, and choroid. Apolipoprotein E and immunoglobulin gamma co-distribute in smaller, hard drusen and in Bruch membrane (as indicated in yellow-green); however, neither protein is present in the spherical core of this drusen. There is some punctate co-localization at the base of the capillary pillar that overlies this drusen (circle). Immunoglobulin gamma is more widespread than apolipoprotein E in the choroidal capillary wall and lumen (CAP). The red-colored granules in the retinal pigmented epithelium (RPE) cytoplasm represent autofluorescent lipofuscin granules, although several larger granules immediately adjacent to the drusen are also likely to be immunoglobulin gamma positive. BM<sub>e</sub> = elastic layer of Bruch membrane. Scale bar = 5  $\mu$ m. (E) Dual-label confocal projection series illustrating the distributions of apolipoprotein E (Cy2 [green] channel) and vitronectin (Cy3 [red] channel) in drusen, Bruch membrane, and choroid. In some larger hard drusen, apolipoprotein E and vitronectin immunoreactivities are detected primarily on the surfaces and basolateral margins. Note that the immunoreactivity at the base of the drusen does not extend into the elastic layer of Bruch membrane. Scale bar = 10  $\mu$ m. (F) Twofold higher magnification of the Bruch membrane region shown in E (small rectangle). Vitronectin and apolipoprotein E co-distribute in the inner collagenous layer of Bruch membrane; vitronectin is not associated with the putative retinal pigmented epithelium basal lamina or with the outer collagenous layer. (G) Twofold magnification of the area enclosed by the larger rectangle in E. In the region immediately below the capillary lumen, apolipoprotein E and vitronectin co-distribute in a distorted inner collagenous layer. In the adjacent region near the capillary pillar, no evidence of Bruch membrane is apparent, and only a cloud of apolipoprotein E/vitronectin-positive particles is visible (arrowhead).

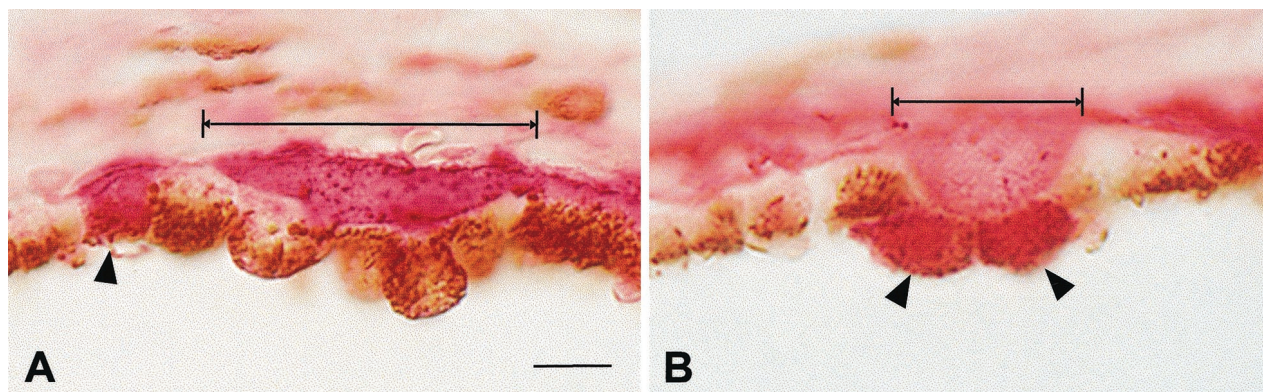


FIGURE 4. Apolipoprotein E immunoreactivity in retinal pigmented epithelial cells that flank and overlie drusen. In those eyes with numerous hard drusen (brackets), retinal pigmented epithelial cells that (A) flank or (B) overlie drusen (arrowheads) often show intense apolipoprotein E immunoreactivity in their cytoplasm. Scale bar = 10  $\mu\text{m}$ .

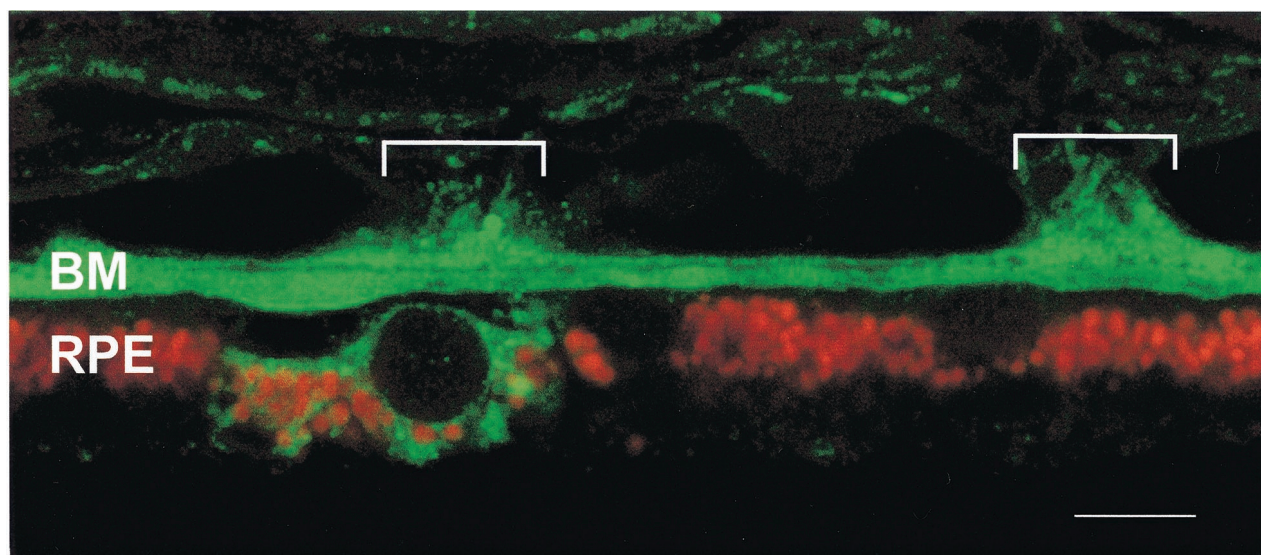


FIGURE 5. Apolipoprotein E immunoreactivity in the retinal pigmented epithelium (RPE) cytoplasm does not co-localize with lipofuscin. Pseudo-dual-label confocal image of apolipoprotein E immunoreactivity (green channel) in the retinal pigmented epithelium/choroid. Immunoreactivity is associated with the capillary pillars (brackets), Bruch membrane (BM), and with granules in the cytoplasm that do not co-localize with autofluorescent lipofuscin granules (red channel). Scale bar = 5  $\mu\text{m}$ .

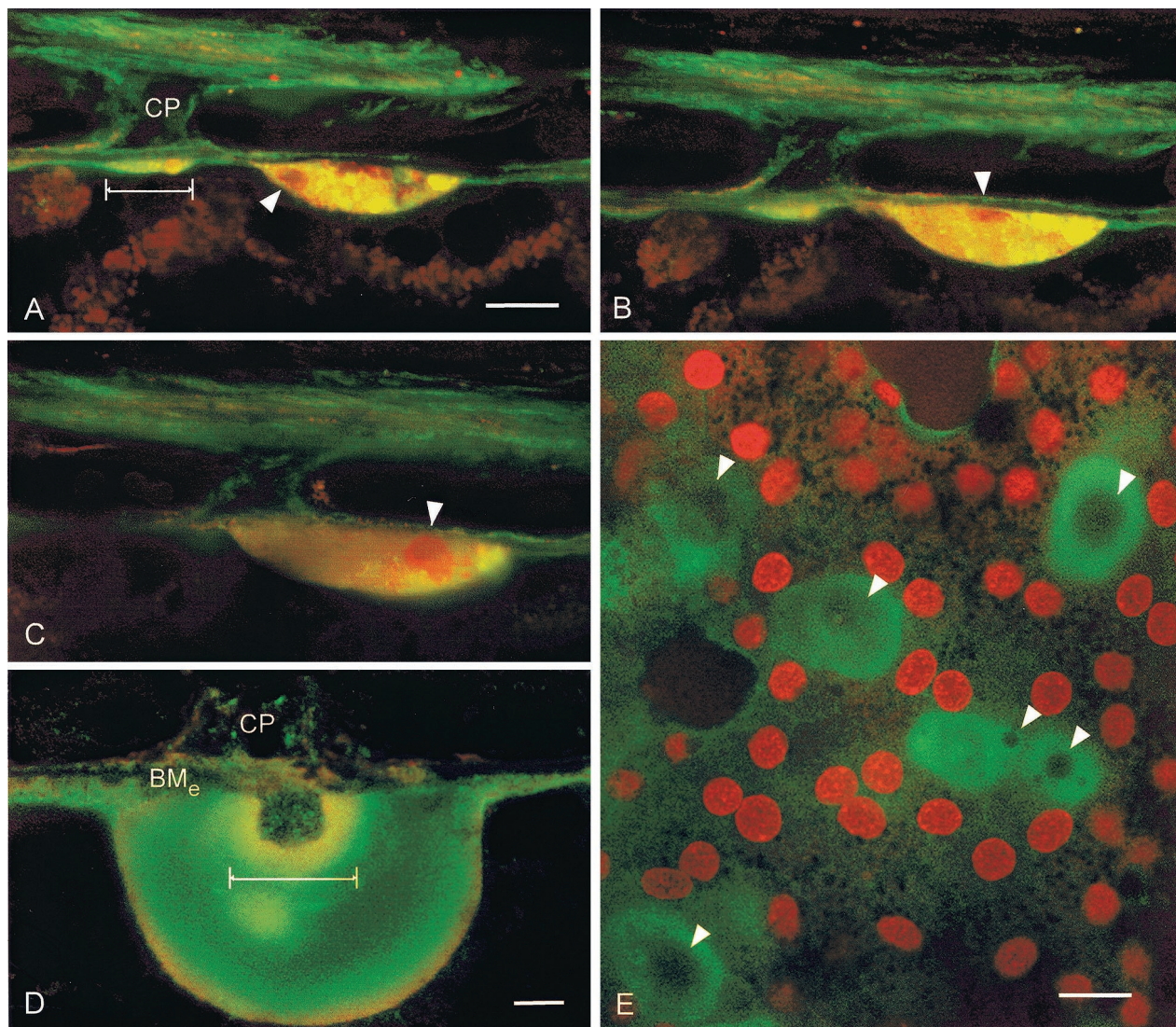
of at least some of these molecules in drusen. Here, we present evidence bearing on the potential biosynthetic contribution of the retinal pigmented epithelium to the apolipoprotein E found in drusen.

Like vitronectin, apolipoprotein E immunoreactivity is found in most, if not all, drusen irrespective of clinical phenotype. Their respective distributions in the retina, in Bruch membrane, and in the choroid overlap to some degree, but they are not identical. The quantitative reverse transcriptase-polymerase chain reaction data appear to rule out the photoreceptors as a significant local source of apolipoprotein E. The levels of apolipoprotein E mRNA in the photoreceptor-enriched retinal fraction, relative to the inner retinal fraction, are less than 1% (Figure 8). There-

fore, the apolipoprotein E immunoreactivity associated with the photoreceptor cells is probably not the result of photoreceptor biosynthesis. It may be produced in the liver and then transported to the photoreceptors by means of the circulation, or contributed by other local cell types.

In contrast to vitronectin, apolipoprotein E synthesis in human brain is much more robust, and represents a significant fraction of the amount synthesized by the liver. Astrocytes are apparently responsible for the bulk of this synthesis.<sup>40,41</sup> Although apolipoprotein E transcripts have recently been localized to neurons in certain regions of the brain,<sup>42</sup> no evidence of apolipoprotein E biosynthesis by retinal neurons has emerged thus far. In the rabbit retina, it is reported that Müller cells synthesize and secrete

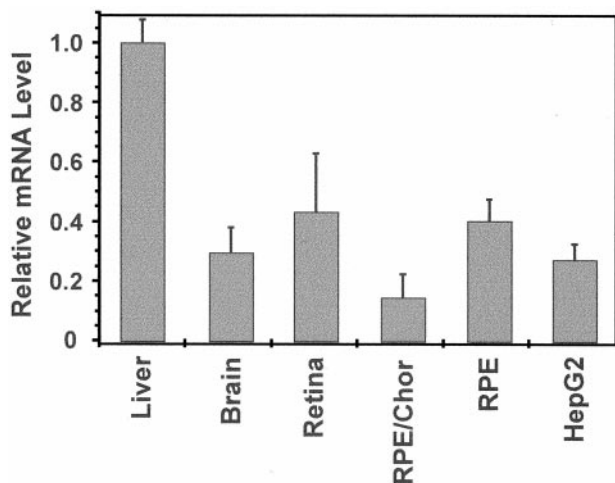




**FIGURE 6.** Dual-label confocal images of apolipoprotein E and vitronectin substructural distributions in drusen. (A to C) Substructural distributions of apolipoprotein E (Cy2 [green] channel) and Vn (Cy3 [red] channel) immunoreactivities in serial optical sections. These drusen contain areas where apolipoprotein E and vitronectin co-localize (shown in yellow), and also separate domains where apolipoprotein E and/or vitronectin are segregated. In A (left), a small vitronectin immunoreactive sphere, which lies immediately below a capillary pillar (CP), appears embedded in an elongated apolipoprotein E-rich zone (brackets). In the larger drusen on the right, a nonimmunoreactive core is enveloped by a “halo” of vitronectin immunoreactivity (A to C, arrowheads). Scale bar = 5  $\mu\text{m}$ . (D) This large drusen consists of a central core with punctate anti-apolipoprotein E immunoreactivity and a concentric ring of apolipoprotein E/vitronectin (bracket). The vitronectin labeling appears to dissipate at increasing distances from the core but then reappears closer to the surface of the drusen. Note that the core of this drusen appears to be continuous with the elastic layer of Bruch membrane ( $\text{BM}_e$ ). Scale bar = 5  $\mu\text{m}$ . (E) En face section at the level of the retinal pigmented epithelium. Sections were labeled with a fluorescent probe (TOPRO-3; red channel) to visualize nucleic acids, and apolipoprotein E antibody (Cy2 [green] channel) to visualize drusen. Intact retinal pigmented epithelium nuclei are intensely stained, but there is no evidence of nucleic acid binding in drusen or in their cores (arrowheads). Scale bar = 10  $\mu\text{m}$ .

apolipoprotein E, which is then assembled into tryglyceride-rich and cholesterol-rich lipoprotein particles that are subsequently internalized by retinal ganglion cells.<sup>43,44</sup> A similar mechanism, involving Müller cell and/or retinal pigmented epithelium biosynthesis, could be responsible for the binding and internalization of apolipoprotein E by photoreceptors.

In both the central and peripheral nervous systems, apolipoprotein E expression by astrocytic glia is upregulated in response to neuronal injury and neurodegenerative disease.<sup>45–47</sup> Similar evidence for apolipoprotein E upregulation by Müller glia has been reported in the degenerating human retina, where increased apolipoprotein E immunoreactivity is found in the subretinal space of detached



**FIGURE 7.** Relative quantitation of apolipoprotein E mRNA in adult human brain, retina, and retinal pigmented epithelium. Estimates of apolipoprotein E mRNA levels were obtained using the real-time quantitative reverse transcriptase–polymerase chain reaction technique described in the text (see Materials and Methods). Mean levels of apolipoprotein E mRNA in the retina are approximately 40% of the level measured in the liver reference standard, and somewhat higher than the apolipoprotein E level measured in brain, a prominent secondary source of apolipoprotein E mRNA. In contrast, mean apolipoprotein E mRNA levels in the retinal pigmented epithelium/choroid are approximately 20% of the amount measured in liver. Apolipoprotein E mRNA levels measured in cultured human retinal pigmented epithelial cells are higher than those measured in the liver-derived HepG2 cell line.

retinas<sup>48</sup> and in Müller cells of retinas affected by glaucoma or age-related macular degeneration.<sup>49</sup> As such, the relatively high levels of apolipoprotein E mRNA measured in the retina, particularly in several of the oldest human donors and in the individual with documented age-related macular degeneration (Table 1), suggest that it may be attributable to increased apolipoprotein E expression by retinal glia.

Based on the results from this and previously published studies, it appears that the Müller cells and the retinal pigmented epithelium are the most prominent biosynthetic sources of apolipoprotein E in the neural retina and retinal pigmented epithelium/choroid respectively. The quantitative reverse transcriptase–polymerase chain reaction data presented here show substantial relative levels of apolipoprotein E transcripts in cultured retinal pigmented epithelial cells as well as in the retinal pigmented epithelium/choroid complex (Figure 7). These quantitative data are reinforced by the immunolocalization results showing apolipoprotein E immunoreactivity on the basal retinal pigmented epithelium surface (Figure 2, A) and in the basal cytoplasm (Figure 2, B).

It is also possible that at least some of the apolipoprotein E in drusen is the result of extravasation from the choroidal

circulation. In that regard, the co-distribution of immunoglobulin gamma and apolipoprotein E within the capillary pillars suggests that this region could serve as an exit pathway of least resistance for plasma proteins leaving the choroidal capillaries, and/or a preferred entry point for macromolecules moving across Bruch membrane in the opposite direction.

Morphologic studies at both the light and electron microscopic level indicate that there is an age-related accumulation of electron dense vesicles that are both basophilic and periodic acid Schiff positive within the capillary pillars, as well as in the inner and outer collagenous layers of Bruch membrane.<sup>50</sup> Immunoelectron microscopic studies in the kidney show that apolipoprotein E is associated with small electron dense granules in the glomerular basement membrane, suggesting that apolipoprotein E-containing lipoproteins or their fragments can cross the glomerular basement membrane and be endocytosed by podocytes as part of their normal housekeeping activities.<sup>51</sup> Moreover, apolipoprotein E is a component of the subepithelial immune deposits that form in passive Heyman nephritis,<sup>52</sup> an experimental rat model of glomerular basement membrane disease in humans.<sup>53</sup> Megalin/gp330, a multiligand receptor from the low density lipoprotein–receptor gene family that binds to and internalizes both apolipoprotein E and apolipoprotein B100, is thought to be the epithelial antigen responsible for the glomerular basement membrane damage that is a hallmark of this disorder.<sup>54,55</sup>

No comparable data with respect to the functional role(s) of apolipoprotein E or its receptors in the retinal pigmented epithelium currently exist, although cultured human retinal pigmented epithelial cells (Hageman GS, unpublished data, 2000) and transformed (SV40) retinal pigmented epithelial cells<sup>56</sup> contain transcripts for a number of lipoprotein receptors. A housekeeping role for the retinal pigmented epithelium in lipoprotein uptake and clearance that is analogous to podocytes is one possible function; a biosynthetic role in lipid transport similar to hepatocytes is yet another. Under either scenario, an age-associated or disease-related functional disruption could result in accumulations of lipoprotein at the interface between the retinal pigmented epithelium and Bruch membrane.

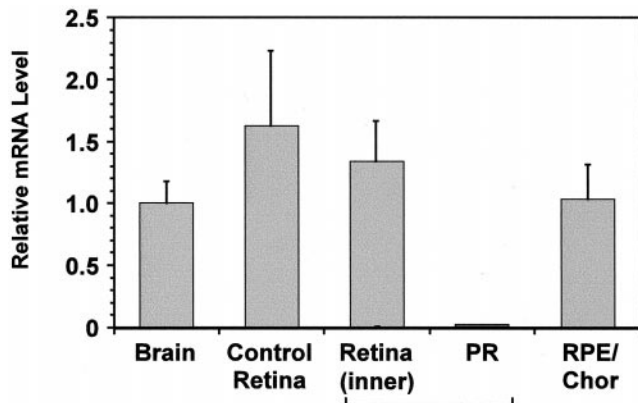
It remains unclear whether the concentration of apolipoprotein E in the cytoplasm of some retinal pigmented epithelial cells, especially those in close proximity to drusen, is the result of biosynthesis or selective accumulation. It is also unclear whether the presence of such cells may be a cause, or a consequence, of drusen formation. Similar cytoplasmic accumulations of immunoglobulin gamma and C5 complement occur in some retinal pigmented epithelial cells of human donors with drusen, and we have hypothesized that this phenomenon is a manifestation of retinal pigment epithelium immune complex pathogenesis.<sup>21,23</sup> Whether the combined pattern of apolipoprotein E, immunoglobulin



**TABLE 1.** Apolipoprotein E mRNA Levels in Human Retina and Retinal Pigment Epithelium/Choroid Relative to Liver

Donor/Gender	Age (years)	Apolipoprotein E mRNA Level*		Fundus Status
		Retina	Retinal Pigment Epithelium/Choroid	
1 Female	34	0.57 ± 0.05	0.09 ± 0.01	No drusen
2 Female	39	0.42 ± 0.03	0.25 ± 0.03	No drusen
3 Male	43	0.36 ± 0.02	0.11 ± 0.01	No drusen
4 Male	46	0.37 ± 0.02	0.10 ± 0.01	No drusen
5 Male	51	0.34 ± 0.04	0.33 ± 0.02	Rare peripheral drusen
6 Male	52	0.24 ± 0.07	0.09 ± 0.04	Few peripheral drusen
7 Male	64	0.33 ± 0.03	0.17 ± 0.02	Few macular drusen
8 Female	66	0.46 ± 0.03	0.18 ± 0.05	No drusen
9 Male	70	0.29 ± 0.02	0.19 ± 0.02	Rare peripheral drusen
10 Male	72	0.32 ± 0.03	0.10 ± 0.01	Few peripheral drusen
11 Female	76	0.29 ± 0.03	0.06 ± 0.01	Moderate peripheral drusen
12 Female	82	0.45 ± 0.30	0.09 ± 0.04	Moderate peripheral drusen
13 Female	89	0.93 ± 0.08	0.17 ± 0.04	Rare peripheral drusen
14 Male	98	0.35 ± 0.02	0.06 ± 0.01	Many peripheral drusen
15 Female	98	0.78 ± 0.06	0.17 ± 0.02	Age-related macular degeneration; geographic atrophy

\*Mean values from 3 runs expressed as a fraction of the liver reference standard ± 1 standard deviation.



**FIGURE 8.** Quantitation of apolipoprotein E mRNA in the inner and outer fractions of the neural retina. Normal adult human retinas were separated into an outer fraction (PR) containing the photoreceptor inner and outer segments, and an inner fraction that contained the remainder of the retina (bracketed). Levels of apolipoprotein E mRNA relative to adult human brain were then quantified in real time using the quantitative reverse transcriptase–polymerase chain reaction assay described in the text. Levels of apolipoprotein E mRNA in the inner retinal fraction were nearly equivalent to the levels measured in total retina (control retina), whereas levels measured in the outer retinal fraction were negligible.

gamma, and C5 immunoreactivities defines a single, functionally compromised retinal pigmented epithelium phenotype is currently under investigation.

It is now recognized that complement activation is associ-

ated with a number of diseases that result in the formation of abnormal extracellular plaques or deposits. These include Alzheimer disease,<sup>57–59</sup> atherosclerosis,<sup>60</sup> glomerulonephritis,<sup>61</sup> and skin elastosis.<sup>62</sup> Although activation of the complement cascade by means of the classical pathway is typically associated with immune complex formation, complement can also be activated directly by endogenous molecules in the absence of an immune response.<sup>63</sup> Significantly, several of these molecules are documented drusen constituents, including C-reactive protein, amyloid P component, and cholesterol.<sup>12,23</sup> Further experimental studies will be required to identify the specific trigger(s) for complement activation in the context of drusen formation, to define the functional roles of apolipoprotein E and other lipoproteins in retinal pigment epithelium metabolism, and to determine if disruptions in lipoprotein processing that may occur as a result of aging or inheritance are indeed linked to the pathogenesis of age-related macular degeneration.

#### ACKNOWLEDGMENTS

The authors would like to express their sincere appreciation to Michelle Staples and William Leitner for their invaluable technical assistance in this investigation.

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