Published in final edited form as:
Exp Eye Res. 2009 June ; 88(6): 1129–1136. doi:10.1016/j.exer.2009.01.019.

Accumulation of extracellular RGR-d in Bruch's membrane and close association with drusen at intercapillary regions

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Abstract

Human retinal pigment epithelial (RPE) cells synthesize an extraneous splice isoform of retinal G protein-coupled receptor (RGR). In this study, we analyzed the exon-skipping variant of RGR (RGR-d) that is found in extracellular deposits. RPE-choroid tissue sections were prepared from postmortem human eyes from donors of various ages. RGR-d was analyzed in drusen and Bruch's membrane by immunohistochemical localization. Extracellular RGR-d is present in most drusen, including hard, soft, confluent and early-stage. Initial drusen formation is known to be preferentially associated with the intercapillary regions of Bruch's membrane. We corroborated this significant association of drusen, including early-stage drusen, with the intercapillary regions. The distribution of extracellular RGR-d in Bruch's membrane differs in old and young donors. In older persons, nodes of concentrated RGR-d accumulate at intercapillary loci, predominantly at the lateral edges of the capillaries of the choriocapillaris. RGR-d loci at the lateral capillary wall appear numerous in old, but not young, donors. Intensely immunostained RGR-d loci can be found at the base of early-stage drusen mounds in the older donors and may precede the formation of these drusen.

Keywords

Retinal pigment epithelium; Bruch's membrane; drusen; RGR; RPE retinal G protein-coupled receptor; RGR-d; exon-skipping isoform of human RGR; choriocapillaris; extracellular deposit

1. Introduction

Abnormal protein deposition in extracellular sites is a feature of the age-related neurological diseases, Alzheimer's disease and age-related macular degeneration (AMD). In AMD, extracellular deposits accumulate over time in Bruch's membrane, the innermost stratum of the

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choroid (Feeney-Burns and Ellersieck, 1985; Killingsworth, 1987). Focal extracellular deposits, called drusen, commonly develop external to the basement membrane of the retinal pigment epithelium (RPE) (Hogan et al., 1971; Guymer and Bird, 2001; Sarks and Sarks, 2001; Hageman et al., 2001). Since large and numerous drusen are believed to be pathogenic (Verhoeff and Grossman, 1937; Gifford and Cushman, 1940; Gass, 1972; Midena et al., 1994; Takamine et al., 1998; Johnson et al., 2003, 2005), the mechanism of formation of drusen deposits is of great interest. To understand the formation of drusen, the lipid, carbohydrate and protein constituents of drusen have been studied extensively (Guymer and Bird, 2001; Hageman et al., 2001; Curcio et al., 2005; Li et al., 2007). Many proteins, or protein epitopes, in drusen have been identified, including ubiquitin (Loeffler and Mangini, 1997), integrins (Brem et al., 1994), tissue inhibitor of metalloproteinase 3 (Fariss et al., 1997), amyloid β (Dentchev et al., 2003; Johnson et al., 2002; Loeffler et al., 1995), vitronectin (Hageman et al., 1999), apolipoproteins (Klaver et al., 1998; Mullins et al., 2000; Anderson et al., 2001; Li et al., 2006), clusterin (Johnson et al., 2001; Sakaguchi et al., 2002), clathrin (Bando et al., 2007), HTRA1 (Yang et al., 2006), complement components (Johnson et al., 2000; Mullins et al., 2000), and other proteins associated with inflammation and immune-related processes (Crabb et al., 2002). Despite the interest in these proteins, it is unclear from what cell type drusen proteins originate and in particular, whether the proteins originate from the RPE.

Among RPE-derived proteins that are deposited into Bruch's membrane is a splice isoform of the RPE retinal G protein-coupled receptor opsin (RGR) (Jiang et al., 1995; Fong et al., 2006). RGR is an intracellular membrane-bound protein that belongs to the family of G protein-coupled receptors (Jiang et al., 1993; Pandey et al., 1994). It is expressed preferentially in the RPE and Müller cells and carries all-*trans*-retinal as its endogenous chromophore in the dark (Hao and Fong, 1999). Upon irradiation, RGR converts the all-*trans* isomer stereospecifically to 11-*cis*-retinal. Studies of RGR knockout mice indicate that RGR is necessary to maintain a normal rate of 11-*cis*-retinal synthesis both in light and in darkness after irradiation (Chen et al., 2001; Maeda et al., 2003; Wenzel et al., 2005). RGR mediates the light-dependent translocation of all-*trans*-retinyl esters to be used as the substrate for renewal of 11-*cis*-retinal (Radu et al., 2008).

Rare mutations in the human *RGR* gene are associated with retinitis pigmentosa (Morimura et al., 1999). A more common mutant form of RGR (RGR-d) results from alternative splicing of human RGR pre-mRNA with the complete deletion of the exon 6 sequence (Jiang et al., 1995). The extraneous mRNA that encodes the RGR-d splice variant has been found in all donors analyzed thus far. RGR-d is synthesized in the RPE, and the protein, or peptide fragment that contains an immunohistochemically detectable RGR-d epitope, is released into the sub-RPE space and deposited into Bruch's membrane (Lin et al., 2007). The synthesis and deposition of RGR-d in Bruch's membrane throughout life is likely to also involve ongoing clearance of the molecule. A failure to clear extracellular RGR-d by degradation or transfer would be predicted to affect the distribution or abundance of RGR-d in Bruch's membrane.

In this paper, we investigate the presence of extracellular RGR-d in drusen and in Bruch's membrane of old and young donors. The initial formation of drusen has been shown to be strongly associated with areas of Bruch's membrane adjacent to choroidal intercapillary regions (Lengyel et al., 2004; Friedman et al., 1963; Müller, 1856). We have corroborated the significant association of drusen, including early-stage drusen, with the intercapillary regions and have observed related accumulations of extracellular RGR-d at these intercapillary loci in the eyes of aged, but not young, individuals.

2. Materials and methods

2.1 Antibody Production

An affinity-purified RGR-d antibody, DE21, was produced as described previously (Fong et al., 2006). The DE21 antibody was generated against a synthetic peptide (GKSGHLQVPALIAK) that corresponds to the unique sequence of human RGR-d at the splice junction of exons 5 and 7. The peptide was conjugated to keyhole limpet hemocyanin for immunization. Rabbit antisera were produced by Cocalico Biologicals, Inc. (Reamstown, PA). The DE21 polyclonal antibody was purified by affinity-binding to immobilized peptide attached to Affi-Gel 10 resin (Bio-Rad, Hercules, CA) and was shown to specifically bind recombinant RGR-d protein (Fong et al., 2006).

2.2 Tissue preparation

Postmortem eyes were obtained from the Doheny Eye and Tissue Transplant Bank (Los Angeles, CA) and the National Disease Research Interchange (NDRI, Philadelphia, PA) (Table 1). Tissues for frozen sections were processed without fixation or were fixed with 4% paraformaldehyde in PBS for 4-6 hr at 4°C. Fixed tissues were infiltrated overnight with 30% sucrose in PBS. The retina/RPE/choroid complex was dissected from the sclera, embedded in OCT compound (Miles, Elkhart, IN) and frozen. The frozen tissues were sectioned with a cryostat at −20°C to a thickness of 5-8 μm, mounted on Superfrost/Plus slides (Fisher Scientific, Pittsburgh, PA) and stored at -80 °C. Tissues for paraffin-embedded sections from donors NR1, NRI-3, NRI-1 and 08-003 were fixed in 4% paraformaldehyde in 0.1 M sodium cacodylate buffer, pH 7.4. Paraffin-embedded sections were heat-treated for antigen retrieval, as described previously (Shi et al., 1996).

2.3 Immunohistochemistry

For immunostaining, all RPE-choroid sections were permeabilized by fixation with cold acetone for 5 min. The sections were then incubated with blocking buffer that consisted of 0.2% dodecylmaltoside, 3% bovine serum albumin, and 5% normal goat serum in PBS. After blocking, the sections were incubated with the DE21 antibody, as described previously (Lin et al., 2007). Immunohistochemical staining was performed with the Impress (Vector Laboratories, Burlingame, CA) peroxidase-based enzyme detection system using the Vector VIP (Vector Laboratories) substrate, according to the manufacturer's instructions. Control slides were treated in the same manner, except that the primary antibody was omitted from the binding buffer, or blocked by pre-adsorption with the RGR-d peptide (GKSGHLQVPALIAK) in PBS for 30 min. The sections were mounted in aqueous Crystal/Mount medium (Biomeda Corp., Foster City, CA), or were dehydrated sequentially with 95% and 100% ethanol, cleared with xylene, and covered with VectaMount Permanent Mounting Medium (Vector Laboratories).

Images were photographed using a Leica DM LB2 microscope system with Spot digital camera and Advanced version 4.0.8 (Diagnostic Instruments, Inc., Sterling Heights, MI) or using a Nikon Optiphot microscope with a Nikon D50 camera. Differential interference contrast microscopy (DIC) was performed on an Olympus BH-2 microscope, and images were photographed with a Nikon D50 camera. Statistical analysis of the association of drusen with intercapillary sites was performed by normal approximation to the binomial test (two-tailed), where the expected proportion was the area of the intercapillary pillars to capillary wall regions along Bruch's membrane.

3. Results

3.1 Accumulation of extracellular RGR-d at intercapillary regions of Bruch's membrane

Tissue sections of the RPE and choroid from normal young donors (ages, 23-15 yrs) and older donors (ages, 96-69 yrs) were probed with the DE21 RGR-d antibody. Specific immunostaining was detected in association with Bruch's membrane or in the basal portion of the RPE cells (Fig. 1). There was a continuum of immunoreactivity along Bruch's membrane in the tissue from older donors, and many loci with intense immunoreactivity were noted (Fig. 1, A-C). These highly immunoreactive sites were found preferentially at intercapillary regions, along the walls of the capillary vessels, and in some cases, in the subcapillary region along the external endothelial wall. Comparable loci with strong immunostaining of RGR-d were consistently absent in the normal young donors (Fig. 1, D-F). The bold immunostaining of the intercapillary regions and capillary walls was missing in the young donors, even after considerable over-development of the immunohistochemical stain that was present in the basal portion of RPE cells.

The intense nodal immunostaining in Bruch's membrane of the older donors may represent highly concentrated or transformed RGR-d at specific sites. As shown definitively by differential interference contrast optics, these highly immunoreactive sites were found at intercapillary regions (Fig. 2). The RGR-d loci were located more precisely at the lateral edges of the capillaries and along the ascending walls of the vessels at many, but not all, of these sites within the plane of section (Fig. 2, A and B). Sites with comparable intense immunostaining in the normal young donor were not seen by differential interference contrast optics (Fig. 2C).

3.2 Formation of drusen at intercapillary regions

Donors with a large number of drusen are likely to have these extracellular deposits at different stages of development, some at very early stages of formation. We found strong positive immunostaining of the RGR-d epitope in all large and small early-stage drusen that were observed. In donor NR1, almost all drusen (17/19, P=0.0006) were located at intercapillary sites (Fig. 3), a finding that is consistent with results described previously (Lengyel et al., 2004;Friedman et al., 1963).

3.3 Early-stage drusen and association with RGR-d loci

Interestingly, in most cases, each druse in donor NR1 was juxtaposed to an RGR-d locus having intense immunostaining (Fig. 3). These RGR-d loci were located at the base of the drusen mounds on the choroidal side of Bruch's membrane. The juxtaposition of RGR-d loci and drusen at intercapillary regions was observed for both large and small hard drusen, including early-stage lesions (Fig. 4).

The early-stage drusen were either small rounded mounds or flat deposits with heights much less than half the height of the epithelial layer (Fig. 4). Like larger focal drusen mounds, the minute early-stage forms and thin flattened areas of immunostaining lying external to RPE cells were associated with the intercapillary regions. Such drusen were easily visualized by immunostaining with the RGR-d antibody in the absence of any other histochemical counterstain. The early-stage drusen, which were made conspicuous by immunostaining, were positioned opposite intensely immunostained RGR-d loci that formed bases for the initial drusen material.

3.4 Accumulation of RGR-d in soft and large confluent drusen

Large soft drusen that stained faintly with the periodic acid Schiff stain were present in donors 08-003, 08-075 and 9708039. Donors 08-075 and 9708039 were patients with macular degeneration. Positive RGR-d staining was observed in separate large drusen and along Bruch's

membrane (Fig. 5). Dysmorphic RPE cells were present below the immunoreactive drusen, and thin areas at the base of some RPE cells were stained intensely. In AMD donor 9708039, positive staining was found in areas of confluent soft drusen (Fig. 5D). Background immunostaining in the choroid was low.

3.5 Immunostaining specificity

The immunostaining of drusen was highly reproducible and specific (Fig. 6, A and B). When the RGR-d antibody was pre-incubated with excess RGR-d peptide antigen (GKSGHLQVPALIAK), the immunostaining of drusen was blocked (Fig. 6, C-E). The immunostaining along the entire length of Bruch's membrane was also eliminated. Small early-stage drusen were no longer stained in the control sections.

4. Discussion

Observable differences in the localized concentration of extracellular RGR-d exist between normal young individuals and older donors with age-related changes in Bruch's membrane. In older donors, the accumulation of extracellular RGR-d becomes concentrated at intercapillary regions. The prominent RGR-d loci are typically located at the lateral edges of the capillaries of the choriocapillaris. These intensely immunoreactive RGR-d loci along the lateral, and as far as the external, walls of the capillaries were not seen in normal young donors.

Like RGR-d loci, the initial sites of drusen deposition are strongly associated with the lateral walls of the choriocapillaris (Lengyel et al., 2004; Friedman et al., 1963). Here, we found that the focal drusen mounds were juxtaposed to discrete sites of RGR-d over-accumulation. Notably, early-stage drusen, as well as well-formed mounds, were positioned across from RGR-d loci on the choroidal side of Bruch's membrane. Since RGR-d loci are more numerous than visible drusen mounds, they would appear to precede the formation of drusen.

It is clear that drusen and RGR-d loci share a common molecular component identifiable by specific immunoreactivity. The DE21 antibody that is directed against the RGR-d splice-site epitope shows strong immunoreactivity for hard, soft, large confluent and early-stage drusen. Although the RGR-d isoform is one of many proteinaceous components of drusen, it is among the few, perhaps the only, drusen-associated protein that is known to be preferentially expressed by RPE cells. Although extracellular RGR-d originates most likely from the RPE, Müller cells also synthesize RGR-d which, if released, could contribute to the deposition of the RGR-d epitope in Bruch's membrane.

The evidence that RGR-d is released into, and perhaps, cleared from Bruch's membrane corresponds to well-known concepts of the continual deposition of material from human RPE and the attendant clearance of basal extracellular deposits from Bruch's membrane. The mechanism by which cellular debris from the RPE is deposited into Bruch's membrane has not been elucidated. The exit of material from RPE may involve budding or evagination of the basal membrane of RPE cells (Ishibashi et al. 1986), exocytosis (Rungger-Brändle et al., 1987), or release of intracellular vesicles such as those found in the basal region of the RPE (Orzalesi et al., 1982). Possibly, the RGR-d protein is degraded in the RPE, and a peptide fragment that contains the RGR-d epitope is secreted or extruded as a waste product under normal conditions.

It is generally believed that debris in Bruch's membrane is cleared through the choriocapillaris. The observation that extracellular RGR-d accumulates at the lateral edge and along the walls of the choriocapillaris suggests that the different sides of the capillary vessels have unique structural and functional properties by which clearance of RGR-d-containing deposits may be mediated. Indeed, the vascular endothelial cell nucleus is generally located on the outer wall,

which is significantly thicker than the internal wall along Bruch's membrane (Bernstein and Hollenberg, 1965). Endothelial cell processes or pseudopodia project from the capillaries toward the RPE and may have structural and functional roles, including the clearance of extracellular debris (Leeson and Leeson, 1967; Matsusaka, 1968; Yamamoto and Yamashita, 1989; Guymer and Bird, 2004). Endothelial cell fenestrations are more numerous in the internal wall than in the lateral wall, and even more so than in the external wall of the choriocapillaris (Bernstein and Hollenberg, 1965). These fenestrations or pores in the endothelial cells allow rapid exchange of fluid and large material into and out of the capillaries. Large proteins and small ions move preferentially through the attenuated internal endothelial wall (Bernstein and Hollenberg, 1965). The polarization and natural asymmetry of the choriocapillaris may account for the initial over-accumulation of extracellular RGR-d at preferred sites near the lateral edges of the capillaries in older eyes with impaired vascular permeability or transformed RGR-d deposits.

The preferential initiation of drusen at the intercapillary portion of Bruch's membrane has been recognized among the earliest descriptions of senile drusen (Müller, 1856). It is possible that the clearance of extracellular RGR-d from Bruch's membrane becomes impaired or blocked in older eyes, which then may result in its accumulation at specific intercapillary regions where the aberrant protein is concentrated or transformed. Although large differences in RGR-d localization between young and old donors were observed, the difference between patients and age-matched normal donors is not yet clear due to the limited sample population in this study. The uninterrupted production and continued release of RGR-d from the RPE may contribute to the growth of the druse mass. Consistent with this notion is the strong presence of RGR-d in different drusen types, including hard, soft and large confluent drusen. The mutant RGR-d offers a novel potential link between the molecular genetics of human RPE and the formation of age-related drusen.

Acknowledgments

The authors thank Meng-Yin Lin and Fred Ross-Cisneros for technical assistance and the Doheny Eye and Tissue Transplant Bank for providing donor eye tissue for this research. This work was supported in part by National Eye Institute grants EY03040 and EY08364 (HKWF), EY11527 and EY17404 (LVJ), Research to Prevent Blindness, and the Gordon and Evelyn Leslie Macular Degeneration Fund.

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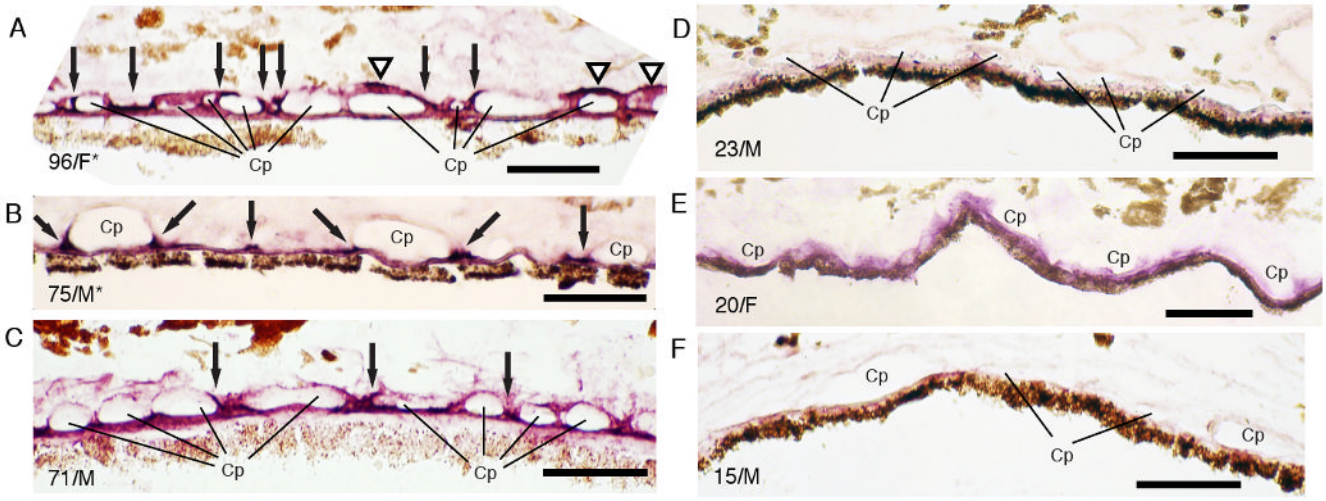

Fig. 1.

Intense immunostaining of extracellular RGR-d at sites in Bruch's membrane of older donors. RPE-choroid sections were obtained from donors (A) 9708039 (96/F), (B) NR1 (75/M), (C) 06030 (71/M), (D) NRI-3 (23/M), (E) Y-005 (20/F) and (F) NRI-1 (15/M) as noted in the lower left-hand corner of the micrographs (* indicates donor's diagnosis with AMD). The tissue sections were incubated with the DE21 antibody and immunostained with the VIP substrate. Arrows point to loci of especially strong immunostaining along Bruch's membrane in the older donors (A-C). The intensely labeled RGR-d loci were located specifically at intercapillary regions in the older donors and were absent in the young donors (D-F). In donor 9708039 (A), intense immunostaining was also observed along the external endothelial wall (*open triangles*) of some vessels. Cp, capillary. Scale bars: 40 μm (each panel).

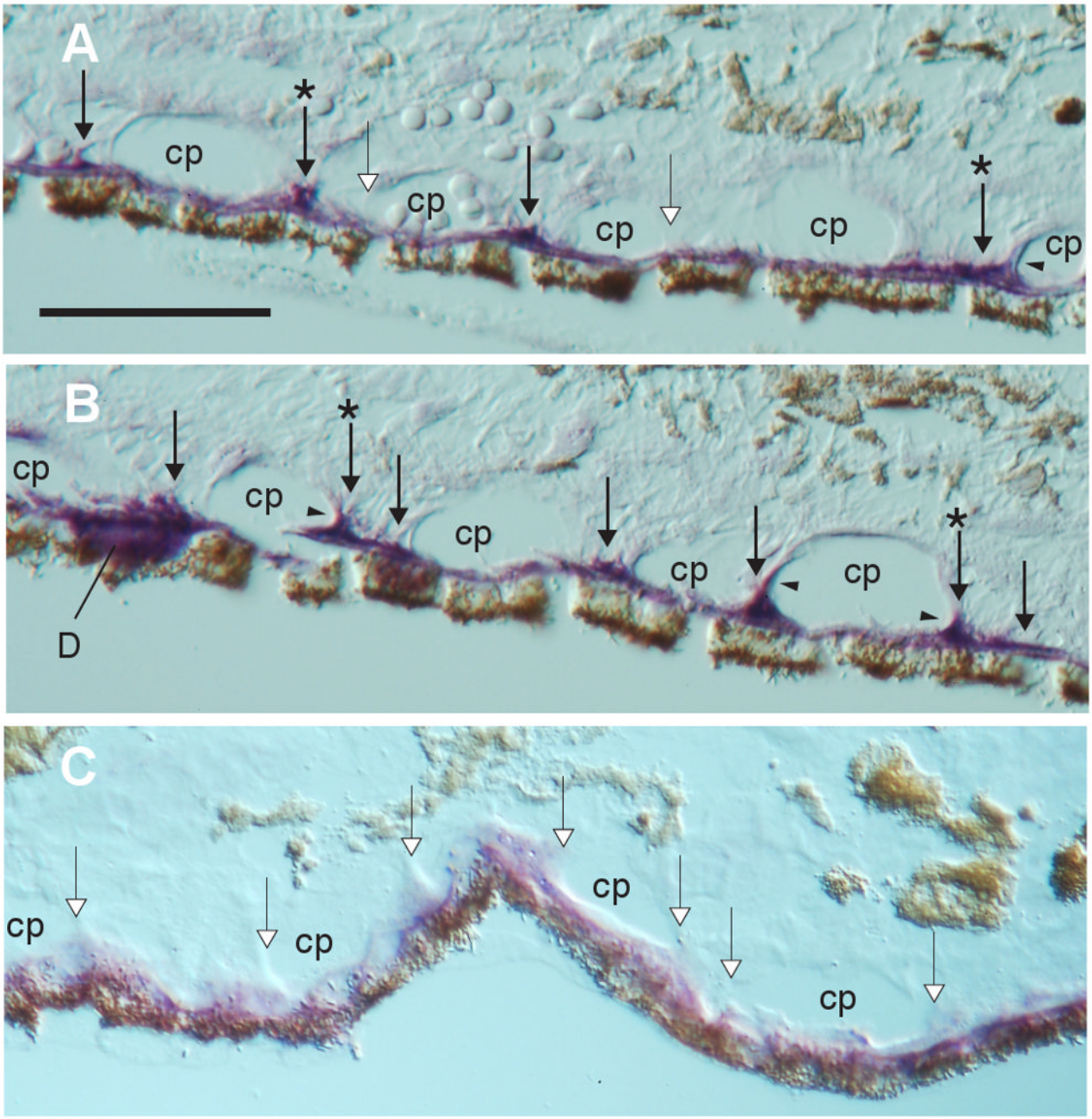

Fig. 2.

Detection of RGR-d loci at intercapillary regions by differential interference contrast microscopy. RPE-choroid sections from old and young donors, NR1 (A, B) and Y-005 (C), respectively, were immunostained with primary antibody DE21. In NR1, highly immunoreactive RGR-d concentration or transformation sites were located preferentially at intercapillary regions (*solid arrows*), at the lateral ends of the capillaries (*solid arrows with asterisks*), along the walls of the capillary vessels (*arrowheads*) and adjacent to drusen mounds. Immunostaining of the intercapillary sites was specific as not all capillary edges were labeled (*open arrows*) in the older donor, and no such labeling was observed in the tissue from the

younger donor (C). Panels B and C overlap Fig. 1B and 1E, respectively. Cp, capillary; D, drusen. Scale bar for panels A-C: 40 μm.

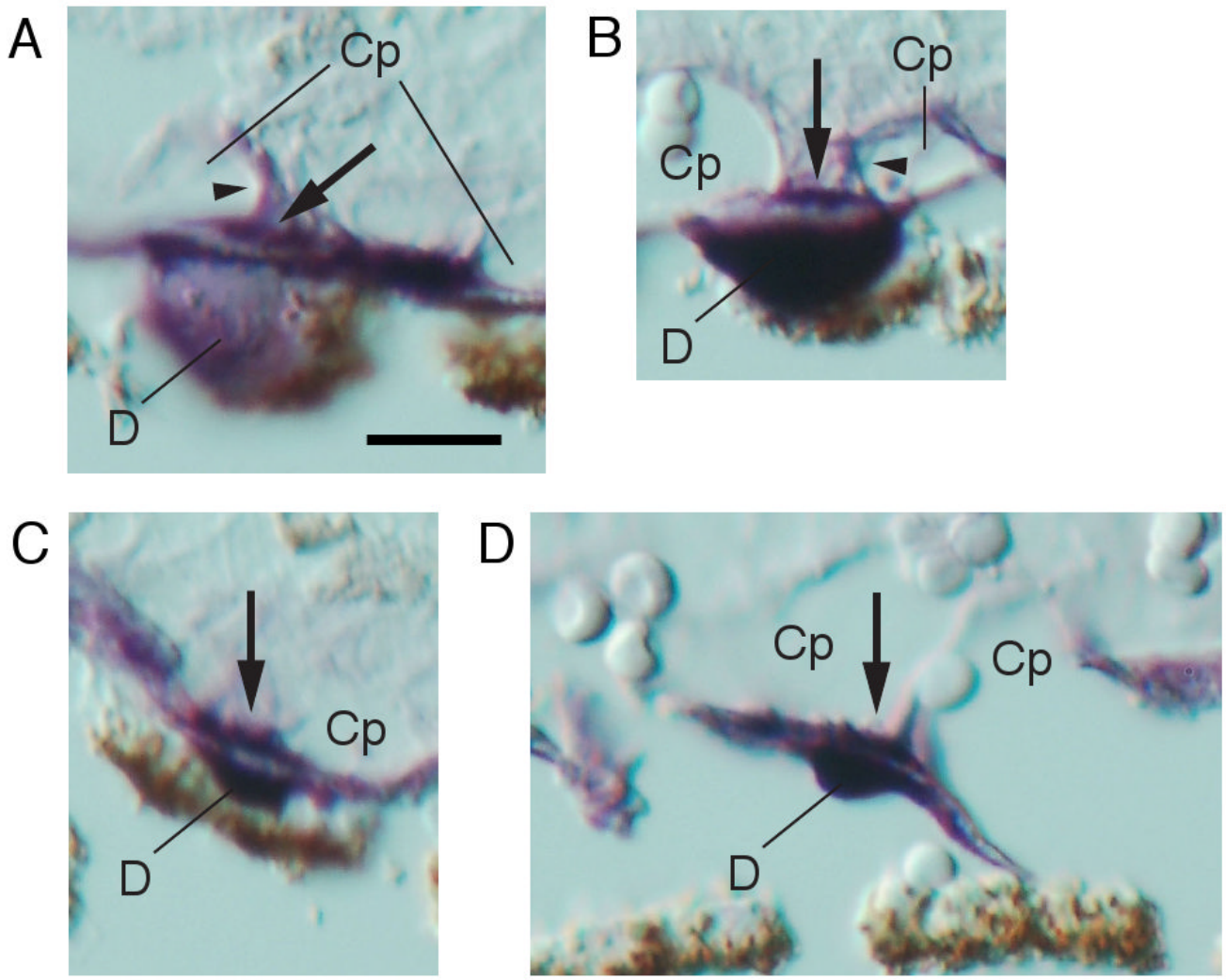

Fig. 3.

Positive immunostaining and the association of drusen and RGR-d loci at intercapillary regions. The RPE-choroid section from AMD donor NR1 was viewed by differential interference contrast optics. The purple VIP chromogen indicates positive staining of drusen (A-D), including small drusen less than the height of an RPE cell (C, D). Intensely immunostained RGR-d loci (*arrows*) were found along the base of each drusen mound. Walls of some capillary vessels were also immunostained (*arrowheads*). Both drusen and RGR-d loci were located at intercapillary regions. Cp, capillary; D, drusen. Scale bar for panels AD: 10 μm.

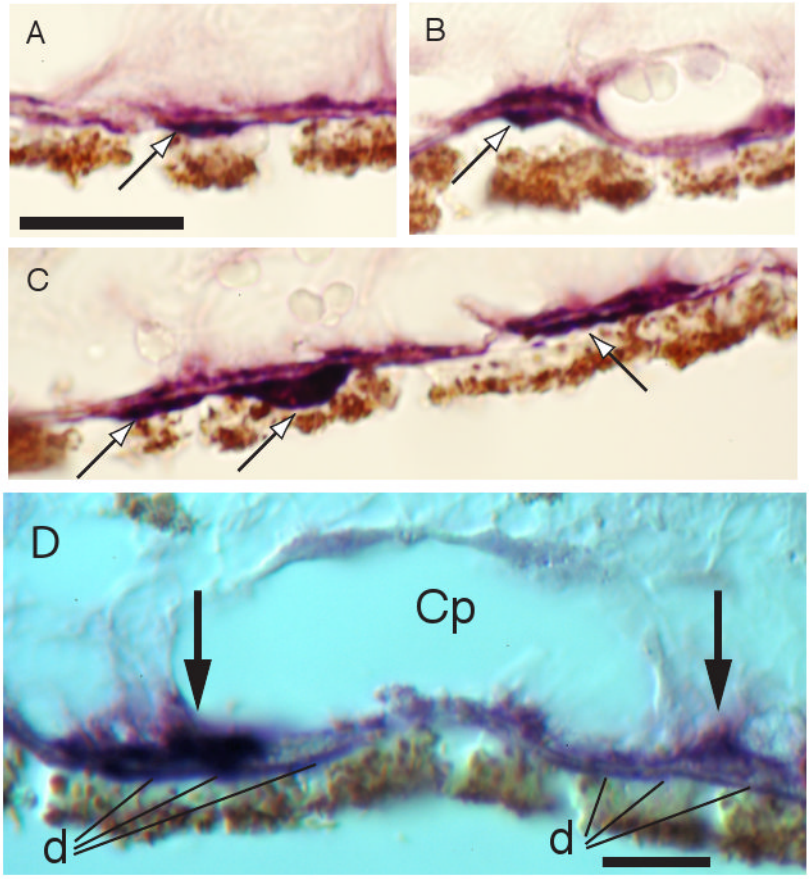

Fig. 4.

Immunostaining of small early-stage drusen. The RPE-choroid section from donor NR1 was prepared from formalin-fixed paraffin-embedded tissue and stained with the DE21 antibody. (A-C) The purple VIP chromogen indicates positive staining of early-stage drusen (*open arrow*). Incipient drusen were stained intensely and were easily seen despite heights less than half those of the RPE cells. (D) Difference interference contrast photomicrograph at high magnification showing intensely stained RGR-d loci (*solid arrows*) at the lateral edge of a capillary (*Cp*). Flat early-stage drusen (*d*) are present between the RPE and the RGR-d loci, and not elsewhere. Scale bars: 20 μm (panels A-C) and 10 μm (panel D).

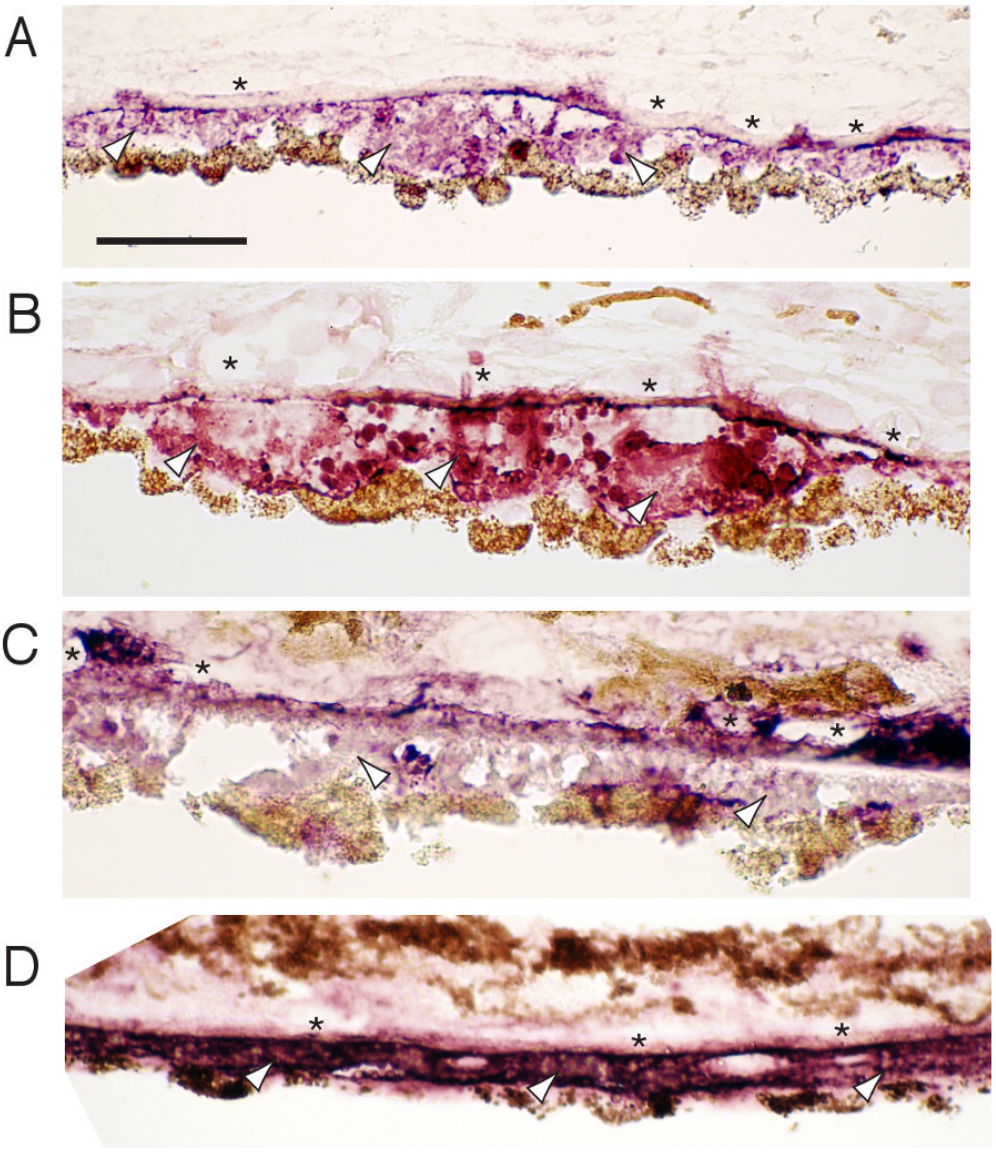

Fig. 5.

Immunohistochemical localization of RGR-d epitope in soft and large confluent drusen. RPE-choroid sections were from (A and B) donor 08-003 (85/M), (C) 08-075 (79/F) and (D) 9708039 (96/F). The VIP chromogen indicates positive staining of four separate large drusen (*open arrowhead*) in the macula (A-C) and peripheral retina (D). Paraffin-embedded sections were treated for antigen retrieval and then probed with the DE21 antibody (A and B). Sections from frozen tissue were incubated with DE21 antibody without antigen retrieval (C and D). Donors 08-075 and 9708039 were patients diagnosed with macular degeneration. The asterisks indicate capillaries. Scale bar for panels A-D: 40 μm.

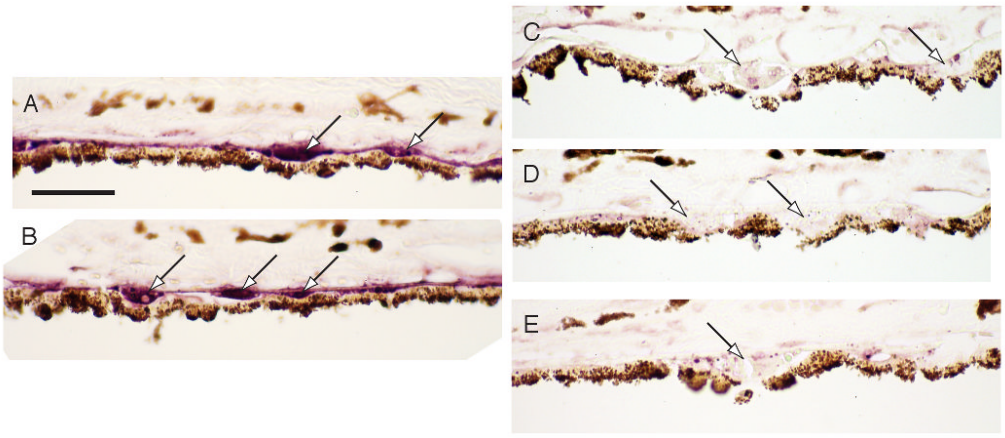

Fig. 6.

Specific immunostaining of drusen by RGR-d peptide antibody DE21. The RPE-choroid sections from AMD donor 960105 were prepared from frozen tissue and stained with the DE21 antibody. (A and B) The purple VIP chromogen indicates positive staining of drusen (*open arrow*). Dysmorphic RPE cells are observed above the larger drusen. (C-E) Control sections were treated identically as in A and B, except that the primary antibody was pre-incubated with peptide antigen at a concentration of 10 μM. Scale bar for panels A-E: 40 μm.

Table 1

Information on postmortem donor eyes

Donor	Age/Gender	Known ocular history[1]
9708039	96/F	MD,
08-003	85/M	none
08-075	79/F	MD
NR1	75/M	MD
06030	71/M	none
960105	69/M	MD
NRI-3	23/M	none
Y-005	20/F	none
NRI-1	15/M	none

[1] Ocular history is from information provided by the eye banks. MD, macular degeneration; none, no known retinal disease.