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An Integrated Hypothesis That Considers Drusen as Biomarkers of Immune-Mediated Processes at the RPE-Bruch's Membrane Interface in Aging and Age-Related Macular Degeneration ☆

Gregory S. Hageman[a,*], Phil J. Luthert[b], N.H. Victor Chong[a,b], Lincoln V. Johnson[c], Don H. Anderson[c], Robert F. Mullins[a]

[a] *Department of Ophthalmology and Visual Sciences, The University of Iowa Center for Macular Degeneration, PFP 11190E, 200 Hawkins Drive, The University of Iowa, Iowa City, IA 52240, USA*
[b] *The Institute of Ophthalmology, University College of London, Moorfields Eye Hospital, London, UK*
[c] *Center for the Study of Macular Degeneration, Neuroscience Research Institute, University of California, Santa Barbara, CA, USA*

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*Corresponding author. Tel.: +1-319-384-9722; fax: +1-319-335-7142; e-mail: gregory-hageman@uiowa.edu

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Abstract — Age-related macular degeneration (AMD) is a blinding disease that afflicts millions of adults in the Western world. Although it has been proposed that a threshold event occurs during normal aging which leads to AMD, the sequelae of biochemical, cellular, and/or molecular events leading to the development of AMD are poorly understood. Although available data provide strong evidence that a significant proportion of AMD has a genetic basis, no gene(s) has yet been identified that causes a significant proportion of AMD. Moreover, no major molecular pathways involved in the etiology of this disease have been elucidated.

Drusen, pathological deposits that form between the retinal pigmented epithelium (RPE) and Bruch's membrane, are significant risk factors for the development of AMD. In our view, the development of testable new hypotheses of drusen origins has been hindered significantly by the absence of a comprehensive profile of their molecular composition. In this review, we describe an integrated ultrastructural, histochemical, molecular biological, and biochemical approach to identify specific molecular pathways associated with drusen biogenesis. The implicit assumption underlying these recent investigations has been that a thorough understanding of the composition of drusen and source(s) of drusen-associated material is likely to provide fresh insight into the pathobiology underlying AMD. Significantly, these studies have revealed that proteins associated with inflammation and immune-mediated processes are prevalent among drusen-associated constituents. Transcripts that encode a number of these molecules have been detected in retinal, RPE, and choroidal cells. These data have also lead to the observations that dendritic cells, potent antigen-presenting cells, are intimately associated with drusen development and that complement activation is a key pathway that is active both within drusen and along the RPE-choroid interface.

We propose herein a unifying hypothesis of drusen biogenesis that attempts to incorporate a large body of new and previously published structural, histochemical, and molecular data pertaining to drusen composition and development. This theory is put forth with the acknowledgment that numerous AMD genotypes may exist. Thus, only some aspects of the proposed hypothesis may be involved in any given AMD genotype. Importantly, this hypothesis invokes, for the first time, the potential for a direct role of cell- and immune-mediated processes in drusen biogenesis.

We acknowledge that the proposed hypothesis clearly represents a paradigm shift in our conceptualization pertaining to pathways that participate in the development of drusen and age-related macular degeneration. It is our hope that other investigators will test, validate and/or refute various aspects of this hypothesis, and in so doing, increase our overall understanding of the biological pathways associated with early AMD.

1. DRUSEN: AN OVERVIEW

Bruch's membrane (BM) lies at the boundary between the ocular retinal pigment epithelium (RPE) and the primary capillary bed of the choroid, the choriocapillaris (Coats, 1905; Hogan *et al.*, 1971; Killingsworth, 1987). This stratified extracellular matrix is comprised of two collagen layers, referred to as the inner and outer collagenous layers, that flank a central domain comprised largely of elastin fibers and elastin-associated proteins. A number of excellent, comprehensive reviews pertaining to the structure, composition and function of Bruch's membrane in normal and diseased eyes have been published recently (Guymer and Bird, 1998; Kliffen *et al.*, 1997; Marshall *et al.*, 1998).

Pathological changes at the level of Bruch's membrane are common findings in aging and age-related diseases (Guymer and Bird, 1998; Marshall *et al.*, 1998). One common change that manifests at this interface is the focal deposition of extracellular material, referred to as drusen, between the basal lamina of the RPE and the inner collagenous layer of Bruch's membrane (Fig. 1). Drusen are important risk factors for, and bioindicators of, age-related macular degeneration (AMD), the leading cause of irreversible blindness in developed countries (discussed in more detail below). In spite of their established association with AMD and a few other ocular conditions, evidence pertaining to their origin and molecular composition has been sparse.

In this review, we provide an overview of the clinical and clinicopathologic literature pertaining to drusen structure. This review is by no means exhaustive, and the reader is referred to the following excellent reviews for additional information and detail (Bressler *et al.*, 1994; Elman and Fine, 1989; Green and Key, 1977; Green *et al.*,

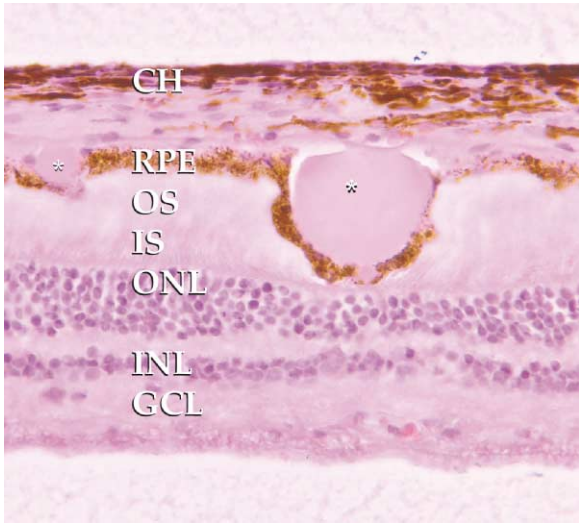


Fig. 1. Light micrograph depicting the appearance and location of "hard" drusen. Drusen are located in the sub-RPE space between the RPE basal lamina and the inner collagenous layer of Bruch's membrane. Note the attenuation of photoreceptor outer segments (OS) underlying the large druse. Asterisks, drusen; CH, choroid; IS, inner segments; ONL, outer nuclear layer; INL, inner nuclear layer; GCL, ganglion cell layer.

1985; Sarks and Sarks, 1989). We focus more specifically upon our current knowledge of drusen composition and origin, including recent data pertaining to the identity of drusen-associated proteins and lipids, a unique association between drusen and dendritic cells, and the potential site(s) of synthesis for a number of drusen-associated molecules. Various theories of drusen biogenesis are discussed, and a working hypothesis of drusen biogenesis that incorporates new information with that of previous studies and observations is advanced. Central to this hypothesis is the principle that inflammatory and/or immune-mediated processes, including the recruitment and maturation of dendritic cells, play a pivotal role in drusen biogenesis and, in the etiology of AMD. This hypothesis introduces a novel paradigm for drusen biogenesis and its relationship to AMD based upon the dynamic interaction between factors that induce and sustain chronic local inflammation, and mechanisms that may have evolved to attenuate it.

1.1. Drusen in aging and age-related macular degeneration

Drusen are most commonly observed in individuals over the age of 60, and in the clinical context of AMD. Their precise role in the pathogenesis of AMD is not clear, although it has long been recognized that drusen are hallmark lesions of AMD, and that, from a clinical standpoint, AMD is rarely diagnosed in the absence of drusen. The size, number, and extent of confluency of drusen are important determinants for the risk of developing AMD (Pauleikhoff *et al.*, 1990). The presence of macular drusen is a strong risk factor for the development of the two predominant clinical forms of AMD, referred to as the atrophic (or "dry") form and the exudative (or "wet") form (Abdelsalam *et al.*, 1999; Bressler *et al.*, 1988b; Sarks, 1980; Smiddy and Fine, 1984). Extramacular drusen appear to be a significant risk factor for the development of AMD as well (Lewis *et al.*, 1986). The presence of soft, large and/or confluent drusen (see below) is correlated to the occurrence of choroidal neovascularization. The Macular Photocoagulation Study Group (1993) has shown that the relative risk for developing choroidal neovascularization (CNV) in eyes that possess five or more drusen is 2.1 and 1.5 in eyes with one or more large drusen.

The presence of drusen is associated with various visual deficits that occur prior to a loss of visual acuity; these include changes in color contrast sensitivity, macular recovery function, central visual field sensitivity, and spatiotemporal contrast sensitivity (Frennesson *et al.*, 1995; Holz *et al.*, 1995; Midena *et al.*, 1997, 1994; Stangos *et al.*, 1995; Tolentino *et al.*, 1994). A few recent studies have shown that visual acuity improves in some cases following laser photocoagulation and subsequent drusen regression (Abdelsalam *et al.*, 1999; Frennesson and Nilsson, 1998; Ho *et al.*, 1999). These observations provide additional support for the notion that there exists a correlation between drusen and vision loss in patients with AMD.

In 1995, the International ARM Epidemiological Study Group developed a classification scheme for grading age-related maculopathy (ARM), which incorporates drusen-based parameters. In

this scheme, ARM is defined as a degenerative disorder in persons 50 years of age or more, characterized on grading of color fundus transparencies, by the presence of soft drusen 63 μm or larger in diameter, hyperpigmentation and/or hypopigmentation of the RPE, RPE and associated neurosensory detachment, (peri)retinal hemorrhages, geographic atrophy of the RPE, or (peri)retinal fibrous scarring in the absence of other retinal (vascular) disorders. Visual acuity is not used to define the presence of ARM (Bird *et al.*, 1995).

1.2. Classification of drusen: clinical and clinicopathologic studies

A vast amount of literature pertaining to the clinical, histological and ultrastructural characteristics of drusen is available. Various schemes have been advanced in an attempt to classify drusen phenotypes, drusen distribution, and the contribution of drusen to the risk of developing AMD (Macular Photocoagulation Study Group, 1997; Bird *et al.*, 1995; Bressler *et al.*, 1994, 1988a, 1989; Curcio *et al.*, 1998; Green, 1999; Green and Enger, 1993; Holz *et al.*, 1994b). Not surprisingly, most studies have concentrated on the funduscopic appearance and morphologic/morphometric features of drusen (Bressler *et al.*, 1994; Green, 1999; Green and Enger, 1993; Spraul and Grossniklaus, 1997). Studies vary according to the nature and extent of clinicopathological correlation, the number of cases examined, the post-mortem interval and the classification system employed. Recent reviews (Bressler *et al.*, 1994; Hageman and Mullins, 1999) detail many of these classification schemes; brief summaries of some of these are provided below.

The Wisconsin drusen grading system, based solely upon the assessment of stereoscopic 30 degree color fundus photographs (Klein *et al.*, 1991), was developed in an attempt to provide consistency to clinical drusen classification. In this scheme, "hard" drusen were defined as discrete drusen between 1 and 63 μm in diameter and drusen were defined as being "soft" if they are larger than 125 μm in diameter, or if they were between 63 and 125 μm in diameter, with "visible thickness". Soft drusen were further categorized as either "soft distinct" (large drusen with uniform density) or "soft indistinct" (large drusen with graded density and fuzzy edges).

Bressler and coworkers have compared various histological and ultrastructural features of drusen with their clinical appearance in the same eyes. They made direct comparisons between funduscopic appearance, fluorescein angiographic features and morphology in two patients with AMD. In this scheme, hard drusen were defined as a function of size (less than 63 μm across) and soft drusen were divided into three distinct categories, based on ultrastructural characteristics (Bressler *et al.*, 1994).

Sarks and coworkers recognized "small hard" drusen, which were discrete and rarely over 125 μm in diameter; drusen derived from the coalescence of drusen (termed "hard clusters," "soft clusters," and "confluent soft drusen derived from clusters"); and "soft membranous" drusen, that resembled confluent soft drusen but were composed of membranous coils. Based on their collective data, Sarks and colleagues proposed that two pathways of drusen formation could be distinguished. They suggested that soft drusen were derived from hard drusen, based on changes in fundus photographs and angiograms taken from the same patients over a number of years. Based on these observations, as well as ultrastructural images of drusen coalescence, they proposed that hard drusen "soften" and coalesce over time (Sarks *et al.*, 1988, 1994, 1980). Sarks and colleagues suggested that the earliest stages in drusen formation may be distinguished between eyes with only a few solitary hard drusen and eyes with large numbers of soft drusen (Sarks *et al.*, 1999).

Spraul and Grossniklaus classified drusen histopathologically as "hard" (small hyaline deposits), "large" (discrete drusen with diameters of greater than 63 μm), "soft" (drusen with pale staining characteristics and sloping borders) or "confluent" (coalescence of 3 or more individual drusen) (Spraul and Grossniklaus, 1997). Green and Enger derived clinicopathological correlations in eleven patients and mapped the pathologic characteristics of a few patients by serial section reconstruction (Green and Enger, 1993).

Recently, Curcio and coworkers have developed a system based on associations between gross

observations of human donor eyes, such as drusen and pigmentary changes, with their histological and ultrastructural features. The classification system derived from these studies is referred to as the Alabama ARMD Grading System (Curcio *et al*., 1998). These authors have also presented data derived from calculations of the sensitivity and specificity of membranous debris (basal linear deposit) for ten age-related maculopathy cases identified using the histopathologic criteria of this system. The data presented suggest that membranous debris is an age-related maculopathy-specific lesion and that basal linear deposits and large drusen should be regarded as the same lesion. These authors suggest that the RPE produces membranous debris and that this material may increase ones risk for developing choroidal neovascularization by generating a cleavage plane within Bruch's membrane (Curcio and Millican, 1999).

The ultrastructural characteristics and relationship between drusen phenotype and composition from over 400 eyes in The University of Iowa Human Donor Eye Repository have been reviewed recently (Hageman and Mullins, 1999). Based upon ultrastructural features, five distinct drusen classes were recognized. Interestingly, no strict relationship between drusen size (one important discriminator between "hard" and "soft" drusen class) and drusen phenotype was noted for four out of the five drusen phenotypes. The immunohistochemical data suggested that different phenotypes of drusen, although they may differ significantly with respect to their substructural morphology, possess a similar complement of proteins and saccharides. It is envisioned that this information will allow investigators to determine whether distinct drusen "phenotypes" exist and whether multiple phenotypes are present in any one individual.

Despite the numerous classification systems employing somewhat different criteria, there is a general consensus about several characteristics of age-related drusen. Clinicians most typically use the terms "hard" and "soft" to describe drusen funduscopically (see Fig. 2). Hard drusen appear as small, punctate, yellow nodules. They generally possess a rounded contour and steeply sloping sides, in contrast to the 'soft' forms that manifest a more gently sloping profile. 'Soft' drusen tend to be larger than 'hard' forms (Bird *et al*., 1995; Bressler *et al*., 1994; Sarks *et al*., 1994). In fundus

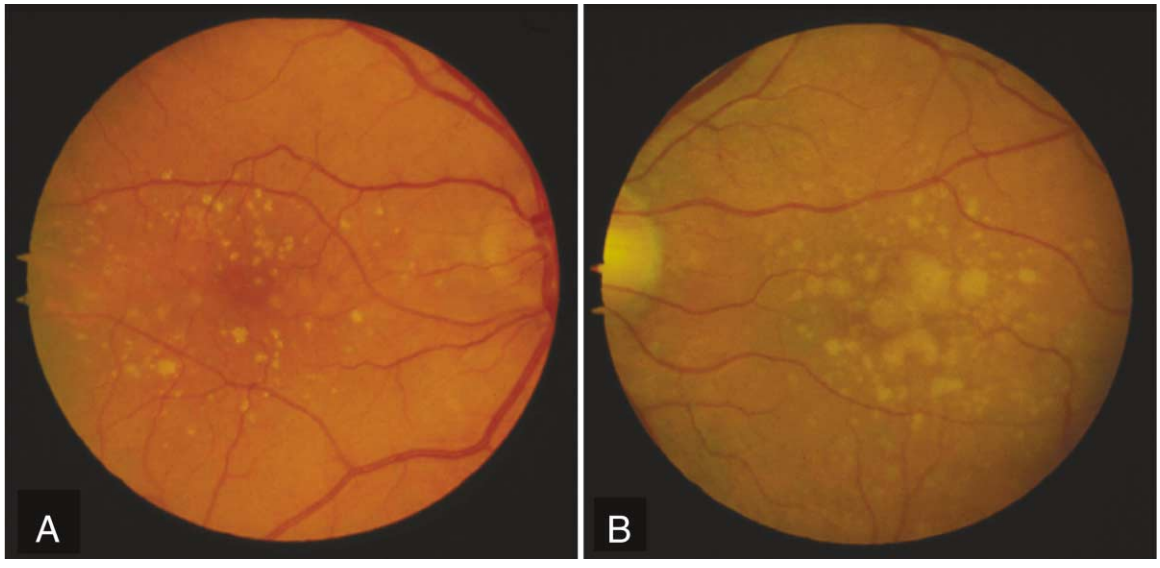


Fig. 2. Comparison of the gross appearance of the two major drusen phenotypes observed at the funduscopic level. "Hard" drusen (A) tend to be smaller with relatively distinct margins, whereas "soft" drusen (B) are larger and typically have less distinct borders. Thirty degree fundus photographs are depicted.

photographs, they appear as large pale yellow or grayish-white, dome-shaped elevations that can resemble localized serous RPE detachments. Moreover, they are often associated with clinically evident RPE detachments and choroidal neovascularization (CNV). Both 'hard' and 'soft' varieties may coalesce, sometimes to the point that they lose clear boundaries and are described as being "diffuse". At this point, the distinction between confluent drusen and "basal linear deposit" blurs. Basal linear deposit consists of a thin, relatively extensive layer of membranous profiles (Green, 1999). A further unifying property is the position of each of these various deposits in relation to the basal lamina of the retinal pigment epithelium. All age-related drusen lie external to the RPE basal lamina. This is in contradistinction to basal laminar deposit, a diffusely deposited material that lies internal to the RPE basal lamina. Basal laminar deposit is heterogeneous at the light microscopic level of resolution, but upon electron microscopic observation is comprised of fibrils, amorphous material, and a banded component that is reminiscent of long-spacing collagen (Marshall *et al.*, 1994). It has been observed that when hard drusen disappear, the overlying RPE and outer retina may become atrophic leading to geographic atrophy.

1.3. Angiographic features of drusen

Most drusen hyperfluoresce early in the angiogram as choroidal fluorescence is transmitted through defects in the overlying retinal pigment epithelium (Pauleikhoff *et al.*, 1992; Scheider and Neuhauser, 1992). Fluorescence from most small drusen diminishes as the dye leaves the choroidal circulation. However, some larger drusen display hyperfluorescence or staining at later stages. The larger the drusen, the more likely it is that they will retain fluorescein and staining will occur. When drusen are large and have smooth edges, the late staining on the angiogram is similar in appearance to that of pooling of fluorescein under a pigment epithelial detachment. In many cases it is difficult, if not impossible, to differentiate large drusen from small pigment epithelial detachments given the similarity of the ophthalmoscopic and fluorescein angiographic features. Indeed, the histopathological features can be similar.

Drusen that do not fluoresce in angiography may be hydrophobic, perhaps due to the presence of neutral lipid that would prevent dye from entering and binding (Pauleikhoff *et al.*, 1992), although differences in the choroid and/or Bruch's membrane associated with some drusen might also explain this pattern. Recently, fundus autofluorescence imaging (Delori *et al.*, 2000; von Ruckmann *et al.*, 1997) and indocyanine green (ICG) angiography (Hanutsaha *et al.*, 1998) have been used in an attempt to determine whether distinct drusen phenotypes can be detected clinically. It will be important to correlate findings employing these techniques with histology.

1.4. Drusen are present in conditions other than AMD

Drusen or drusen-like deposits also develop in young individuals in a few conditions. They often appear over pigmented nevi (Naumann *et al.*, 1966) and malignant melanoma (Fishman *et al.*, 1975). In some cases, the drusen coalesce and form a single, large, hypopigmented lesion (Deutsch and Jampol, 1985). In both situations, these drusen exhibit ophthalmoscopic and fluorescein angiographic features similar to those of age-related drusen. Drusen are also described in longstanding serous retinal detachment, although they are more difficult to detect clinically. Although they have not been studied to the same extent as age-related drusen, the available evidence suggests they are morphologically similar, with 'hard' forms predominating. They are, however, frequently spherical in cross-section, lacking the flattened base typical of drusen observed in aging. Adjacent to choroidal melanomas there is often a region of serous or exudative retinal detachment and drusen-like structures develop here also. Such drusen are not specific for retinal detachments associated with choroidal melanomas, but may be seen in many instances of longstanding retinal detachment. In the main, they appear hyaline and homogenous by light microscopy but differ from age-related forms in that they are often very large. In some instances, massive, aggregates of drusen are observed (Luthert and colleagues, observation; see Fig. 3).

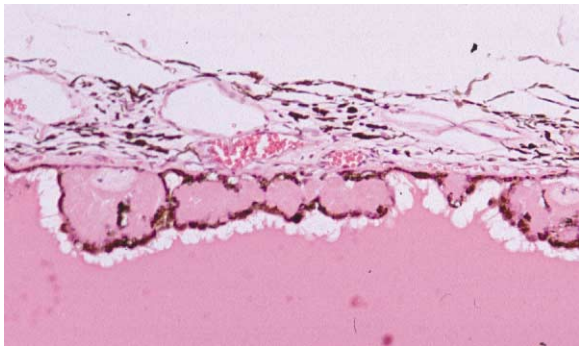


Fig. 3. Light micrograph of a hematoxylin and eosin stained section depicting the RPE and choroid under a region of long-standing retinal detachment. Clusters of eosinophilic drusen-like structures are visible between the RPE and choroid.

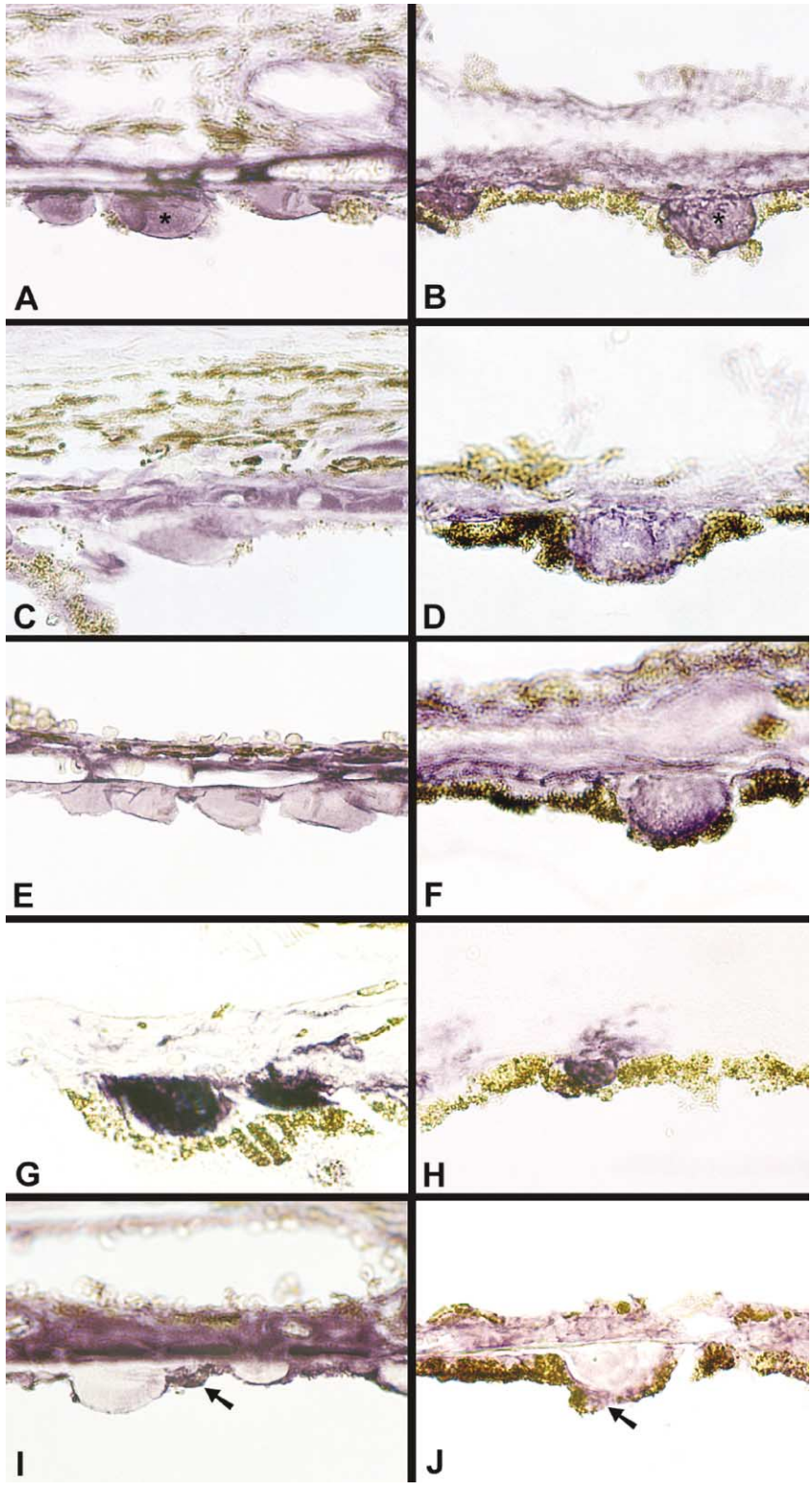
Drusen are also associated with membranoproliferative glomerulonephritis type II (D'Souza *et al.*, 2000; Duvall-Young *et al.*, 1989a,b). These drusen have ophthalmoscopic and fluorescein angiographic features similar to small hard, age-related drusen. However, they tend to be evenly distributed over the entire posterior pole (Leys *et al.*, 1991). This observation is of particular interest from a pathogenic standpoint, and may suggest that drusen in Bruch's membrane and abnormal deposits associated with the glomerular basement membrane could arise from a common or related systemic defect. In addition, a recent study has shown that ocular drusen develop at a relatively early age in other types of glomerulonephritis and that these deposits are morphologically and compositionally similar to drusen in aging and AMD (Mullins *et al.*, 2001).

Rarely, a large number of very small, nodular drusen are observed and the term cuticular or basal laminar drusen has been applied to this funduscopic pattern of pathology (Gass *et al.*, 1985). Histologically, numerous, small, nodular thickenings of the inner aspect of Bruch's membrane are seen (Gass *et al.*, 1985). It has recently been shown that these basal laminar drusen are, like other age-related drusen, located between the RPE basal lamina and the inner collagenous layer of Bruch's membrane, and possess the same molecular composition as age-related drusen (Russell *et al.*, 2000; Fig. 4).

Drusen-like deposits are also observed in some familial macular degenerations, including Malattia Leventinese, or Doyne's Honeycomb Dystrophy (Evans *et al.*, 1997; Piguet *et al.*, 1995), and Sorsby's fundus dystrophy (Polkinghorne *et al.*, 1989). In patients with Malattia Leventinese, brownish-yellow or white drusen-like structures can typically be observed clinically during the third decade of life, and sometimes earlier. By the fourth and fifth decade, the posterior pole is covered by many round, sharply defined white dots, which may be arranged in a mosaic or honeycomb pattern. Some of these deposits are elongated in a radial configuration. They are also present at the optic nerve head and nasal to the optic disc (Piguet *et al.*, 1995). In patients with Sorsby's fundus dystrophy, the drusen start to appear in the second and third decade of life. They are very similar in appearance to age-related drusen but they tend to be uniformly distributed in the posterior pole and can extend beyond the arcades. In the fourth or fifth decades of life, their size increases with increasing distance from the fovea. Sometimes, the drusen in the posterior pole are so densely packed that they are difficult to distinguish ophthalmoscopically, but those in the arcades can be clearly visualized in most of these cases (Polkinghorne *et al.*, 1989).

1.5. Some structures resembling drusen on gross clinical examination are structurally or compositionally dissimilar to age-related forms

It is important to note that some deposits that are similar in appearance to age-related drusen upon clinical examination are distinctly different when they are examined histopathologically. For example, the histological features of the deposits associated with inherited conditions such as Sorby's fundus dystrophy differ significantly from those typically observed in age-related drusen, although they appear similar upon clinical examination. Morphologically, sheets of finely granular or amorphous material are observed within the inner collagenous zone of Bruch's membrane and in places it appears to condense into banded material with a periodicity of 90–110 nm. Where this abnormal extracellular material forms a layer of uniform thickness, it is not visible clinically.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Deposits resembling drusen clinically most probably correspond to focal accumulations of the same substance (Chong *et al*., 2000). Additionally, the structure (Dusek *et al*., 1982; Streicher *et al*., 1982) and composition (Hageman and colleagues, unpublished observations) of the deposits associated with Malattia Leventinese/Doyne's Honeycomb Dystrophy are also markedly distinct from those associated with aging and AMD. In other instances, what appear to be macular drusen clinically cannot be identified upon histological examination of the same eye. In one such case, Frank and colleagues found only small, focal detachments of the RPE histologically in the macula from a donor who had a long clinical history of prominent macular soft drusen (Frank *et al*., 1973).

The observation that distinct deposits detectable on clinical gross examination can give the same appearance as "drusen" suggests that studies on animal models for early AMD must consider the histopathological, as well as the macroscopic, features of these deposits. It is pertinent here to note that data from studies of monkeys (Hirata and Feeney-Burns, 1992; Mullins and Hageman, 1997) and mice (Hawes *et al*., 2000) indicate that drusen-like deposits in animals may differ markedly in both their structure and composition in comparison to drusen associated with AMD in humans.

2. RECENT PROGRESS ON DRUSEN COMPOSITION

A number of studies have been undertaken in order to determine the composition of drusen over the last century and a half, due to the contention that understanding the composition of a disease-related deposit will provide new information about the disease process itself. Some of these studies are reviewed below.

2.1. Lipid components of drusen

The presence of lipid in drusen was inferred by Donders (1854) in the mid-19th century, who noted that drusen (which he originally termed "Colloidkugeln") were often rich in fat ("fettreichen"), based upon the appearance of their small, spherical inclusions. More recent investigations have revealed that drusen are histochemically reactive with such lipid-binding probes as Oil red O, a stain for neutral (i.e., non-polar) lipids (Wolter and Falls, 1962), and Sudan black B, a general lipid stain (Pauleikhoff *et al*., 1992). In a classic set of studies on drusen ultrastructure and composition, Farkas and associates (Farkas *et al*., 1971b) evaluated drusen composition using a combination of enzymatic and histochemical techniques, leading them to the conclusion that the lipid component of drusen is likely to be comprised of the glycolipid moieties, cerebrosides and/or gangliosides. More recently, Pauleikhoff *et al*. (1992) suggested that distinct classes of drusen may exist, with some being more "hydrophilic" and others more "hydrophobic" in character. Holz *et al*. (1994a) compared the macular and peripheral distribution of different lipid classes in Bruch's membrane using chromatographic methods, and concluded that there is a greater relative concentration of lipids in the macular than peripheral regions of Bruch's membrane and that the character of these lipids suggests that they are of cellular, rather than vascular, origin. In contrast to these findings, recent studies by Curcio and colleagues (Curcio *et al*., 2001) described the presence of both esterified and unesterifed cholesterol in Bruch's membrane and drusen, using a combination of histochemical

Fig. 4. Comparison of antibody reactivity to basal laminar drusen (left column) and to drusen associated with aging and AMD (right column). Antibodies directed against vitronectin intensely bind both types of drusen (A, asterisk; B, asterisk). Those directed against complement C5 (C, D) serum amyloid P component (E, F), and HLA-DR (G, H) label drusen associated with both conditions. Antibodies directed against transthyretin (I, J) bind weakly to both drusen types but intensely label the RPE (arrows). (Reproduced from The American Journal of Ophthalmology Vol. 129, Russell et al., Location, substructure, and composition of basal laminar drusen compared with drusen associated with aging and age-related macular degeneration, p. 209 (2000), with permission from Elsevier Science.)

and biochemical analyses. These authors suggest that the presence of large amounts of esterified cholesterol in drusen may imply a vascular (i.e., plasma-based) source for drusen/Bruch's membrane-associated lipids. Data from hot stage polarizing microscopy corroborate the presence of cholesteryl esters in drusen (Haimovici *et al.*, 2001).

Although a number of elegant studies have been conducted, the source of drusen-associated lipids remains an enigma. There is a need for lipid analyses on drusen deposits isolated from human donor eyes, to persuasively determine whether the lipid components of these deposits arise from cellular membranes, the vasculature, or a combination of these sources. Interestingly, in our work on local sources for drusen-associated molecules (discussed below), it has become clear that the genes for some lipoprotein components of drusen (such as apolipoprotein E) are at least transcribed locally, whereas other molecules (such as amyloid P component) appear to have a hepatic origin, being delivered to Bruch's membrane through the vasculature. Drusen associated lipids may have similar, heterogeneous origins.

2.2. Carbohydrate components of drusen

As described above, in an early study Farkas *et al.* concluded that glycolipids were present within drusen (1971b). With the development of newer techniques, the specific sugar chains present within drusen have been evaluated. Kliffen *et al.* (1994) examined Bruch's membrane associated carbohydrates in paraffin-embedded tissue and detected few drusen-associated carbohydrates, although galactosamine-rich glycoconjugates were labeled in basal laminar deposits. Although drusen exhibit periodic acid-Schiff (PAS) reactivity (Farkas *et al.*, 1971b), suggesting the presence of polysaccharide components, Kliffen *et al.* (1996) also evaluated the presence of glycosaminoglycan chains within Bruch's membrane, and found little evidence for chondroitin sulfate or heparan sulfate in drusen.

We have identified glycoconjugates possessing terminal glucose/mannose, *N*-acetylglucosamine, and sialic acids as major drusen carbohydrate moieties (Mullins *et al.*, 1997). Notably, this profile was found to be consistent in drusen of different clinical phenotypes (i.e., hard and soft) (Mullins *et al.*, 1997). One unexpected finding from studies conducted on drusen-associated carbohydrates was that galactosamine disaccharides, not detected in untreated sections, are detectable within drusen following removal of terminal sialic acid residues with neuraminidase (Mullins and Hageman, 1999). This labeling was confined to central, "core" domains within drusen, and appeared to be due to the presence of glycoproteins with O-glycosidically linked carbohydrates (Fig. 5). We have proposed that the presence of core domains within drusen are indicative of a role for cores in the earliest stages of drusen development, similar to the accretion of a pearl around a grain of sand in an oyster's shell. The implications for cores in

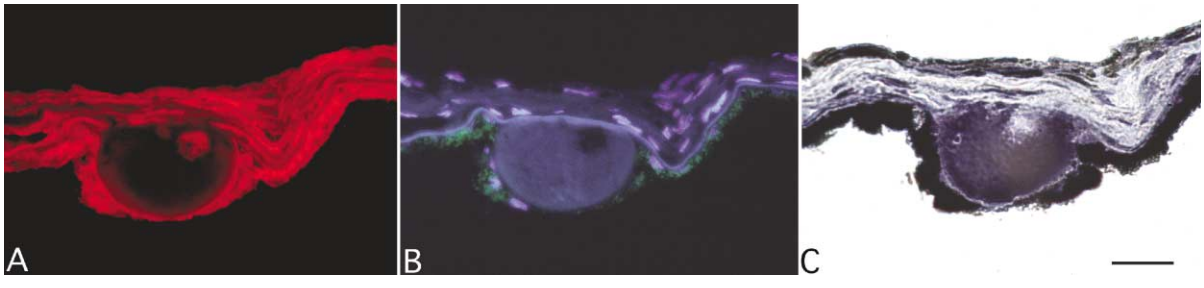


Fig. 5. Serial sections of a druse labeled with rhodamine-conjugated peanut agglutinin after neuraminidase treatment (A), DAPI/UV autofluorescence (B), and Sudan black B (C). Neuraminidase treatment of a druse exposes a PNA binding core (A). When the same section is viewed under UV filters, most autofluorescence of the druse is confined to the material around the core. Nuclear staining of the core with DAPI is not noted (B). Serial sections incubated with Sudan black B (C) reveal staining of the material surrounding the core, suggesting that drusen cores constitute relatively hydrophilic domains. Bar = 30 μm. (Reproduced with permission from Mullins and Hageman (1999), Human ocular drusen possess novel core domains with a distinct carbohydrate composition, The Journal of Histochemistry and Cytochemistry, vol. 47, p. 1537).

drusen development, and the recent insights into the possible synthetic identity of these cores, are discussed below.

2.3. Protein components of drusen

During the last three decades, there have been a surprisingly small number of studies devoted to the characterization of the proteinaceous components of drusen. And, in some cases, conflicting data have been reported. (Newsome *et al*., 1987; van der Schaft *et al*., 1993). Small sample size, different immunolocalization methods and antibodies, variation in post-mortem interval, the use of different fixatives and embedding media—in addition to actual potential differences in composition between different drusen phenotypes—are all possible explanations for these apparent discrepancies. Although such problems cannot be eliminated completely, it is feasible to reduce them significantly by (a) maximizing the numbers of tissue specimens and human donors eyes examined; (b) minimizing death to fixation time; (c) using multiple, well-characterized antibodies; and (d) employing appropriate positive and negative controls.

Drusen constituents identified previously using immunohistochemical methods include ubiquitin (Loeffler and Mangini, 1997), integrins (Brem *et al*., 1994), tissue inhibitor of metalloproteinase 3 (Fariss *et al*., 1997), advanced glycation end-products (Ishibashi *et al*., 1998), beta-amyloid (weak) (Loeffler *et al*., 1995), fibronectin in some phenotypes (Pauleikhoff *et al*., 1992) and C1q (weak) (van der Schaft *et al*., 1993).

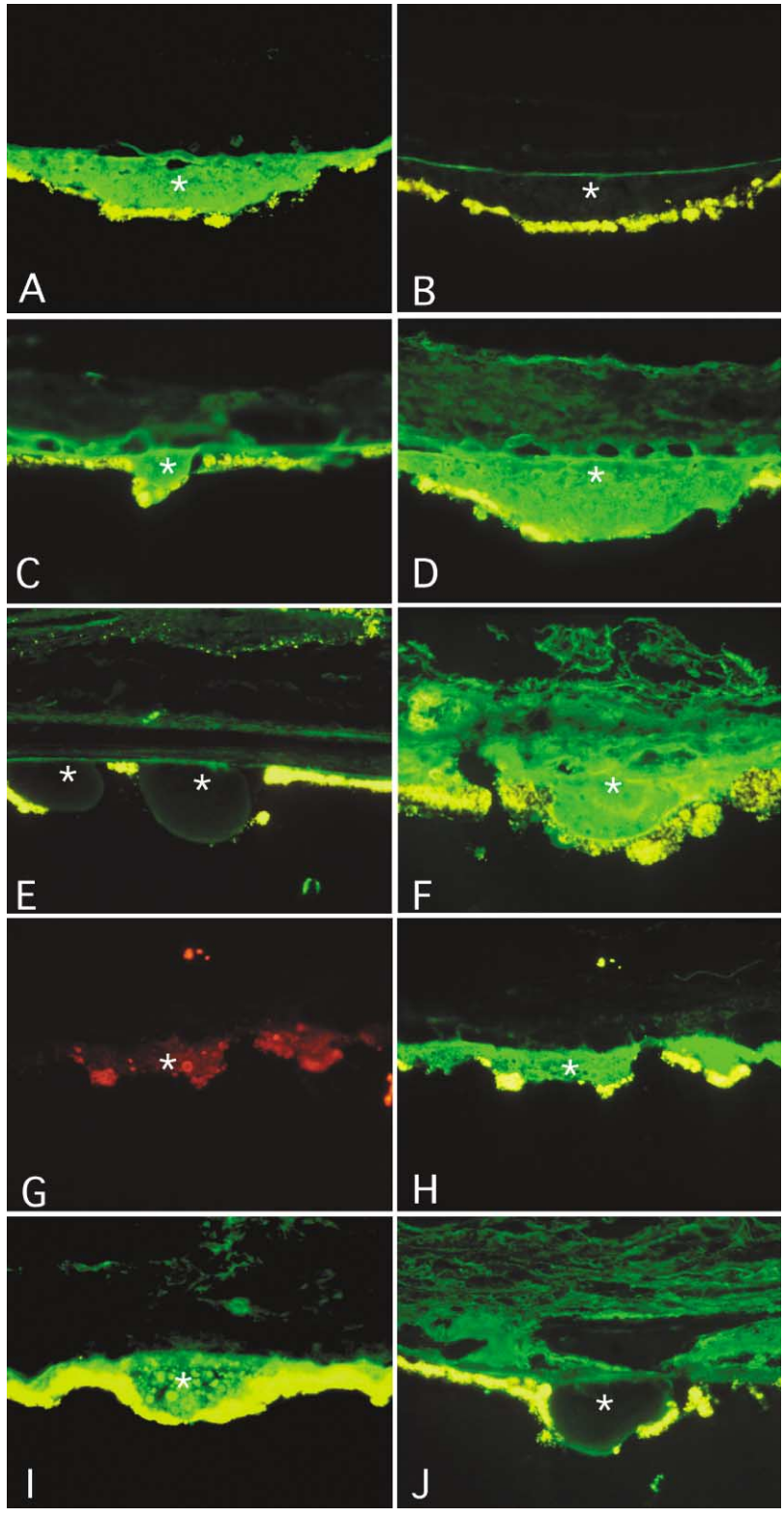
During the last several years, extensive studies directed toward cataloging as completely as possible the proteins associated with drusen and toward determining whether drusen with differing ultrastructural appearances also differ with respect to composition have been conducted. These studies have identified vitronectin (Hageman *et al*., 1999) and a number of molecules associated with extracellular deposits from non-ocular diseases as major drusen constituents. The latter category of molecules includes amyloid P component, apolipoprotein E, factor X, immunoglobulin lambda chains (in some cases), late stage, activated complement components, including the C5b-9 complex, and MHC class II antigens (Johnson *et al*., 2000; Mullins *et al*., 2000a; Mullins and Hageman, 1997) (Fig. 6). Whereas some drusen-associated molecules were not identified consistently, the heterogeneity in labeling does not appear to correspond to any ultrastructural or clinically defined phenotype (Hageman and Mullins, 1999).

Virtually all of the newly-identified proteins in drusen are in some way associated with inflammation and/or other immune-associated processes. Some are classic acute phase reactants, whereas others are components of the complement cascade, or fluid phase inhibitors of the membrane attack pathway of complement. Still others are associated with immune activation, coagulation, and fibrinolysis. Moreover, many of these molecules are common to the pathological deposits associated with other diseases including Alzheimer's disease, atherosclerosis, elastosis, amyloidosis, and glomerulonephritis (Mullins *et al*., 2000a), thus raising the possibility that common pathogenic pathways may be involved in their formation.

2.4. Cellular components of drusen

According to traditional models of drusen formation, any cellular material residing within drusen is predicted to be of RPE origin. Indeed, RPE-derived basal laminae, organelles and cellular fragments, and even entire cells can be detected in early "drusen" (Fig. 7). In addition, blebs of basal RPE that extend through the RPE basal lamina and into drusen or pre-drusen sites has been described (Ishibashi *et al*., 1986). RPE constituents, such as lipofuscin and melanin, are sometimes observed within small, early drusen, where they likely contribute to drusen volume and formation.

One novel and potentially quite significant observation is that cell-associated molecules, including HLA-DR and specific CD antigens, are associated with drusen. These molecules are often localized to solitary, core-like domains within drusen (Mullins *et al*., 2000b). Recent immunophenotyping analyses have documented that these cores are derived from cellular extensions of choroidal dendritic cells, potent antigen presenting cells associated with a variety of immunomodula-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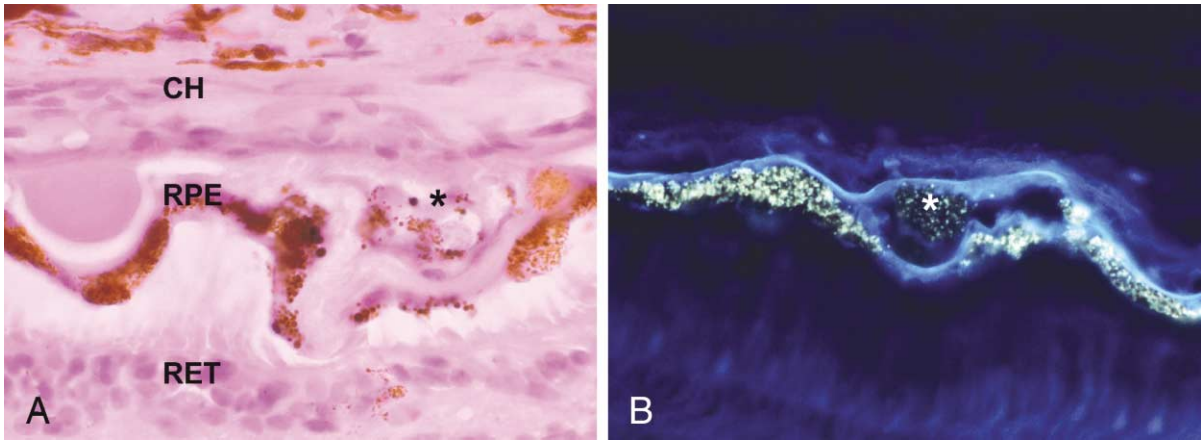

Fig. 7. Light micrographs depicting the presence of autofluorescent lipofuscin/pigment granules within the body of small drusen. These images provide support for the concept that RPE cells contribute to drusen formation and mass. (A) hematoxylin and eosin stained section showing RPE material within a druse. (B) UV autofluorescence demonstrating RPE lipofuscin within a small druse.

tory processes. Moreover, these dendritic cell processes are typically associated with the RPE blebs described above. These data suggest, for the first time, that the process of drusen biogenesis may be cell-mediated and that specific immune-mediated pathways may play significant roles in these events. The implications of these findings with respect to drusen biogenesis are discussed later in this review.

2.5. mRNAs for many drusen-associated molecules are transcribed by local ocular cells

Many of the drusen-associated molecules identified recently using immunohistochemical methods (Hageman *et al.*, 1999; Johnson *et al.*, 2000; Mullins *et al.*, 2000a) are known to be synthesized primarily in the liver. Accordingly, their extravasation from the choroidal capillaries, and their subsequent aggregation along Bruch's membrane, constitutes one potential pathway for their deposition into drusen. However, local cell types in the neural retina, RPE, and/or choroid that lie in close proximity to Bruch's membrane may also have the capacity to synthesize a number of these molecules. Thus, ocular cell types that lie in close proximity to drusen could serve as local cellular sources for some of these drusen-associated molecules. If these local cell types synthesize and secrete significant quantities of drusen-associated molecules, they could make a substantial biosynthetic contribution to the protein content in drusen, and thereby participate in the sequence of microenvironmental events that culminate in drusen development.

Recently, evidence of gene transcription for a number of drusen-associated molecules in the neural retina, RPE/choroid, and several derivative cell lines has been obtained using the reverse

Fig. 6. Fluorescence light micrographs depicting examples of drusen (asterisks) immunoreactivity to various antibodies (see Table 1 for complete profile). Immunolabeling is green (red in panel G) and RPE autofluorescence is yellow. Drusen immunoreactivity is consistently observed with anti-C5 antibodies (A); a secondary antibody control is depicted in panel B. Both small, "hard" (C) and large, "soft" (D) drusen also react with antibodies to apolipoprotein E. Labeling was not generally observed with C1q (E) and albumin (J) antibodies (see also ref 32). Heterogeneous labeling is noted with a number of antibodies that exhibit variable drusen immunoreactivity, including anti-fibrinogen (F), which occasionally labels concentric rings within drusen, prothrombin (G), and amyloid A (I), which label spherical profiles within some drusen. Double-labeling with an anti-vitronectin antibody (H), which labels drusen homogeneously, reveals that vesicular labeling of prothrombin(G) does not correspond to an overall condensation of drusen constituents, but to actual heterogeneous distributions of some drusen-associated molecules. (Reproduced with permission from Mullins *et al.* (2000), The FASEB Journal, Vol. 14, p. 839).

transcriptase polymerase chain reaction (RT-PCR), real-time quantitative RT-PCR, and microarray analyses. In studies employing RT-PCR endpoint analysis, PCR products for a number of drusen associated mRNAs, including TIMP-3 (Alexander *et al.*, 1990), apolipoprotein E (ApoE), vitronectin (Vn), C3, C5, and C9 complement were detected in neural retina, RPE/choroid, and/or isolated human RPE cells (Anderson *et al.*, 2001; Hageman *et al.*, 1999; Mullins *et al.*, 2000a).

Although data derived from RT-PCR analyses have been informative, they provided no information about the levels of expression of a specific molecules. In order to determine whether various drusen-associated molecules are expressed locally in significant quantities and whether the expression of the genes encoding these molecules changes substantially as a function of age, injury, or ocular pathology, quantitative studies, including quantitative RT-PCR (QRT-PCR) and gene array analyses, have been undertaken. For example, quantitative RT-PCR analyses have determined the levels of message for three drusen associated proteins, ApoE, Vn, and C5, in the retina, RPE, and choroid. The normalized ratios of ApoE mRNA in retina and RPE/choroid relative to liver were 0.45 and 0.15, respectively, and the ratios of vitronectin mRNA in retina and RPE/choroid compared to liver were 0.47 and 0.06, respectively. Remarkably, mean retinal mRNA levels for both ApoE and Vn were nearly 50% of those measured in liver, and significantly higher than the ratios in brain relative to liver (0.28 and 0.01). The ratios of C5 mRNA relative to liver were 0.45 ± 0.55 and 0.14 ± 0.12 in retina and RPE/choroid, respectively. In a small series of adult human donors ranging in age from 34 to 98 years, the mean C5 mRNA levels were noted to be considerably higher (by nearly seven fold) than in adult human brain, and about 30% of the value obtained in adult human liver. Mean C5 mRNA levels in the RPE/choroid were not significantly different from those measured in brain, and are substantially lower in RPE/choroid relative to liver (15%). C5 mRNA levels in cultured human RPE cells are approximately equivalent to those found in the liver-derived HepG2 cell line and are nearly 60% of those measured in human liver. Thus, to the extent that the levels of C5 mRNA measured in cultured cells are indicative of their expression *in vivo*, the RPE probably accounts for the majority of C5 mRNA in the RPE/choroid tissue complex (Fig. 8).

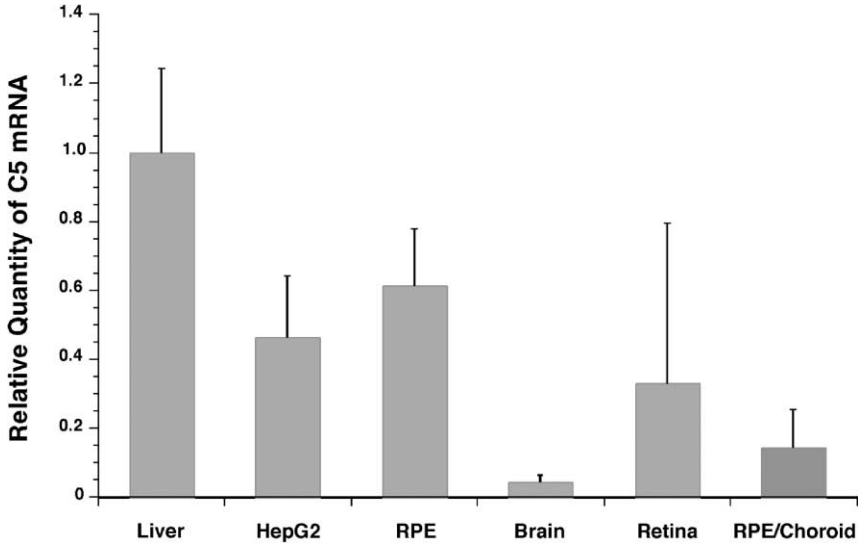

Fig. 8. Quantitative RT-PCR demonstrating relative messenger RNA levels of the complement component C5. Relative quantities of liver (the primary source of complement for the circulatory system), the hepatocyte cell line HepG2, cultured RPE cells, brain, neural retina, and combined RPE-choroid layers. Note the relatively high levels of ocular C5 message in ocular tissues, suggesting that locally synthesized complement molecules may play a role in complement activation within Bruch's membrane and/or drusen.

The relative abundances of these mRNAs in the neural retina and RPE/choroid suggests that local cellular sources in these tissues have the potential to make a substantial biosynthetic contribution to the vitronectin, ApoE, and C5 protein content in drusen and Bruch's membrane. Although these results suggest a possible local source for drusen-associated proteins, one must keep in mind the remote possibility that genes for these molecules are transcribed but not translated. Thus, absolute certainty of the biosynthetic source(s) of these molecules may only be obtained in the rare cases in which the hepatic and ocular genotypes of a single individual are different.

In addition to the patterns of gene expression identified by RT-PCR analyses (above), recent data derived from gene array analyses are yielding novel information concerning the expression and abundance of specific drusen associated molecules. These studies have verified local expression of numerous drusen-associated constituents, including participants in the complement pathway(s), by RPE and/or choroidal cells. Moreover, they have provided quantitative data pertaining to levels of expression as a function of AMD disease state. Although ongoing studies are being directed toward evaluating AMD-associated changes in gene expression levels, it is clear from the combined analyses described above that the mRNAs encoding many drusen constituents are synthesized by ocular cell types.

3. DRUSEN BIOGENESIS

It is almost certain that diverse biological processes are involved in the etiology of AMD. Any hypotheses that are advanced to explain the development of this disease must acknowledge that there may be more than one AMD genotype. Drusen formation, however, is a consistent feature of all AMD phenotypes and probably, all AMD genotypes. Indeed, the presence of small subclinical drusen is typically observed (both clinically and histologically in fellow eyes of donors where one eye exhibits clear evidence of advanced pathology) prior to more advanced pathology in individuals with AMD. Our data suggest that the number of small, subclinical drusen (and associated RPE pathology), especially in the peripheral retina, may be much higher in younger individuals than has been appreciated previously.

In our view, the development of testable new hypotheses of drusen origins has been hindered significantly by the absence of a comprehensive profile of their molecular composition. The implicit assumption underlying recent investigations is that a more thorough understanding of the source(s) of a material (i.e. drusen) can be greatly facilitated by identifying its molecular "fingerprint". A thorough understanding of drusen composition and biogenesis, therefore, is likely to provide fresh insight into the biological events underlying AMD that is not dependent upon prior identification of the causative gene(s) or environmental factor(s) that contribute to it. Using this approach, the cellular sites of synthesis for a number of these components, including vitronectin (Anderson *et al*., 1999; Hageman *et al*., 1999), apolipoprotein E (Anderson *et al*., 2001) and various complement components (Mullins *et al*., 2000a) have been ascertained recently (discussed above). Moreover, large scale RPE and choroid gene expression analyses have revealed not only local synthesis, but also differential expression, of a number of drusen-associated molecules. These combinatorial approaches directed toward understanding the origin(s) of drusen components—and by extension of drusen themselves—are beginning to shed new light on the biological pathways involved in drusen biogenesis and early AMD.

In this section, theories of drusen biogenesis are reviewed in light of these more recent findings. In particular, some of the transitional cell-mediated events that we believe are involved in drusen formation are discussed. Collectively, these data re-affirm the pivotal roles of RPE and dendritic cells in drusen biogenesis and, for the first time, implicate immune-mediated and inflammatory events as integral components of that process. Based upon all available data, we introduce a new paradigm for drusen biogenesis and its relationship to AMD. This integrated hypothesis is based largely upon the dynamic interactions between those factors that induce and sustain chronic local inflammation at the level of the RPE-Bruch's membrane-choroid interface, and those mechanisms that attenuate it.

3.1. Overview of theories of drusen biogenesis: a review of the literature

Although drusen were first described nearly 150 years ago, the cellular and molecular events involved in their formation have not been fully elucidated. Numerous "pathways" for drusen genesis have been postulated in the literature (Duke-Elder and Dobree, 1967; Ishibashi *et al.*, 1986; Wolter and Falls, 1962). These fall into two general categories based on whether drusen are derived from the RPE or the choroid. Theories related to the derivation of drusen from RPE cells include the concepts that drusen result from: secretion of abnormal material derived from RPE or photoreceptors ("deposition theories"—Ishibashi *et al.*, 1986; Muller, 1856; Young, 1987); transformation of degenerating RPE cells into drusen ("transformation theories"— Donders, 1854; El Baba *et al.*, 1986; Fine, 1981; Rones, 1937); or some combination of these pathways. These two hypotheses of drusen biogenesis were referred to by Coats (Coats, 1905) as the "transformation" and "deposition" theories, respectively.

Donders, who first described drusen in a post-mortem eye, believed that drusen were derived from RPE nuclei, based on the supposition that the latter are relatively resistant to degradation. Donders' theory was later modified by De Vicentis (1887) who proposed that degenerative change in the RPE cytoplasm, rather than in the nucleus, was the precipitating event. On the other hand, Muller (1856) proposed that drusen result from aberrant secretion of basement membrane components by the aged RPE. Alt described execrescences in Bruch's membrane as rare sites for ocular bone deposition, and noted that the RPE is intact only over the smallest of drusen (Alt, 1877). Rudnew (1871) proposed that migration and degeneration of leukocytes within Bruch's membrane contribute to drusen formation. On the basis of his own observations, Coats argued that, in their earliest stages, drusen appear to be "simple localized bulgings" of Bruch's membrane that bear no resemblance to degenerating or transformed RPE cells. He did not find histological evidence of definite transitional forms from the degenerating cellular to the acellular phenotype. He concluded, therefore, that drusen are probably a manifestation of a defect in the RPE's biosynthetic contribution to Bruch's membrane.

With the advent of electron microscopy, the substructural features of drusen were revealed, and new variants of the earlier theories were advanced. Some investigators have concluded, based on ultrastructural data, that drusen are formed when the RPE expels portions of its basal cytoplasm into Bruch's membrane (Ishibashi *et al.*, 1986), possibly as a mechanism for removing damaged cytosol (Burns and Feeney Burns, 1980) or as a byproduct of phagocytic degradation (Young, 1987). Not all investigators, however, have been able to detect this phenomenon (Sarks *et al.*, 1999). Others have postulated that drusen are formed by autolysis of the RPE, due to aberrant lysosomal enzyme activity (Farkas *et al.*, 1971a), although enzyme histochemical studies failed to demonstrate the presence of lysosomal enzymes in drusen (Feeney-Burns *et al.*, 1987). Additional mechanisms for drusen formation, including lipoidal degeneration of the RPE (El Baba *et al.*, 1986; Fine, 1981), have been proposed. Friedman *et al.* (1963) proposed that drusen are derived from vascular sources based upon the distribution of drusen near collecting venules. That drusen are distributed near collecting venules was later confirmed in studies by McLeod and Lutty (1994).

Duke-Elder and Dobree (1967) concluded that both the transformation and deposition theories are correct, and that both processes can occur in the same eye (Rones, 1937). They also suggested that theories relating to a choroidal origin of drusen should be disregarded.

Many hypotheses that have been advanced to date, however, are highly speculative, are not based upon rigorous, definitive data derived from large sample sizes, and are not based on information gathered from multiple approaches (i.e., ultrastructure, immunohistochemistry, and molecular biology). More recent investigations have employed an integrated approach to address issues related to drusen composition and biogenesis. Based upon all available data, the putative sequence of pathogenic events culminating in drusen deposition appears to involve elements from RPE transformation, vascular deposition, and leukocyte migration and activation. As such,

we propose that these theories should not be regarded as being mutually exclusive.

4. RECENT DEVELOPMENTS IN DRUSEN BIOGENESIS

Due to the lack of data pertaining to molecular composition, prior hypotheses of drusen biogenesis have relied almost exclusively upon morphologic observations and criteria. The addition of new compositional data, however, can now be used to formulate new hypotheses aimed at identifying the putative sequence of pathogenic events associated with drusen development.

4.1. The relationship between the RPE and drusen biogenesis

From a clinical perspective, AMD is typically associated with pigmentary abnormalities that are attributed to the RPE. Furthermore, the RPE associated with drusen often appears compromised histologically. However, the precise involvement of the RPE in drusen biogenesis has not been characterized rigorously. In addition, although it is generally accepted that RPE density decreases with age, little information is available concerning the density of the RPE in association with AMD and drusen grade. It is unclear if drusen (or other abnormal changes in the extracellular environment in the vicinity of Bruch's membrane) are a cause, or a consequence, of RPE dysfunction. An accumulation of drusen could cause local interference with the exchange of metabolites and waste products between the choriocapillaris and an otherwise normal RPE, leading to RPE dysfunction and death. On the other hand, drusen may develop as a consequence of RPE cell dysfunction caused by a variety of microenvironmental and/or genetic influences. Various hypotheses have been advanced to explain the mechanisms of RPE dysfunction in aging and AMD, including gene mutations, oxidative insults, lipofuscin accumulation, light damage, and others. Whatever the progression of pathological events, RPE dysfunction and degeneration most surely leads to a concomitant degeneration of the underlying photoreceptor cells which, in turn, explains the progressive loss of central vision that is characteristic of AMD.

There are a number of direct and/or indirect lines of evidence supporting a role for the RPE in drusen biogenesis. Some authors have described the appearance of RPE "debris" blebbing into drusen or pre-drusen sites (Ishibashi *et al.*, 1986). We have confirmed this event in ultrastructural studies employing hundreds of human donor eyes. However, it is typically not observed in association with larger, "mature" drusen, but rather, in regions of Bruch's membrane where smaller, "early" drusen appear. In addition, some RPE constituents, including basal laminae, as well as lipofuscin and melanin granules, are observed within such drusen, providing evidence that they probably contribute to drusen volume and formation (Fig. 7). The observation that the RPE synthesizes mRNAs for a number of drusen associated molecules at relatively high levels also supports a role for the RPE in some areas of drusen biogenesis. Finally, processes from choroidal dendritic cells (see below) are typically associated with RPE blebs, fragments, and debris. We have also noted that RPE cell loss is correlated with increasing drusen density (Fig. 9), which is consistent with the notion that RPE pathology is a primary event in drusen formation and growth. Alternatively, a decrease in RPE cell density may be a secondary event that is attributable to the "toxic" effect of overlying drusen.

4.2. Immune-mediated processes and drusen biogenesis

Data from a number of laboratories provide compelling evidence that inflammatory and/or immune-mediated events may participate in the development of drusen and/or progression of AMD. Readers are referred to a review by Penfold and colleagues for a recent discussion of the role of immunological aspects of AMD focused on areas other than drusen biogenesis (Penfold *et al.*, 2001). Accumulations of giant multinucleated cells (Dastgheib and Green, 1994; Penfold *et al.*, 1986) and other leukocytes (Killingsworth *et al.*, 1990; Penfold *et al.*, 1985) in the choroid of donors with AMD have been noted, though not thoroughly quantified, and HLA-DR immunoreactivity of retinal microglia increases in AMD (Penfold

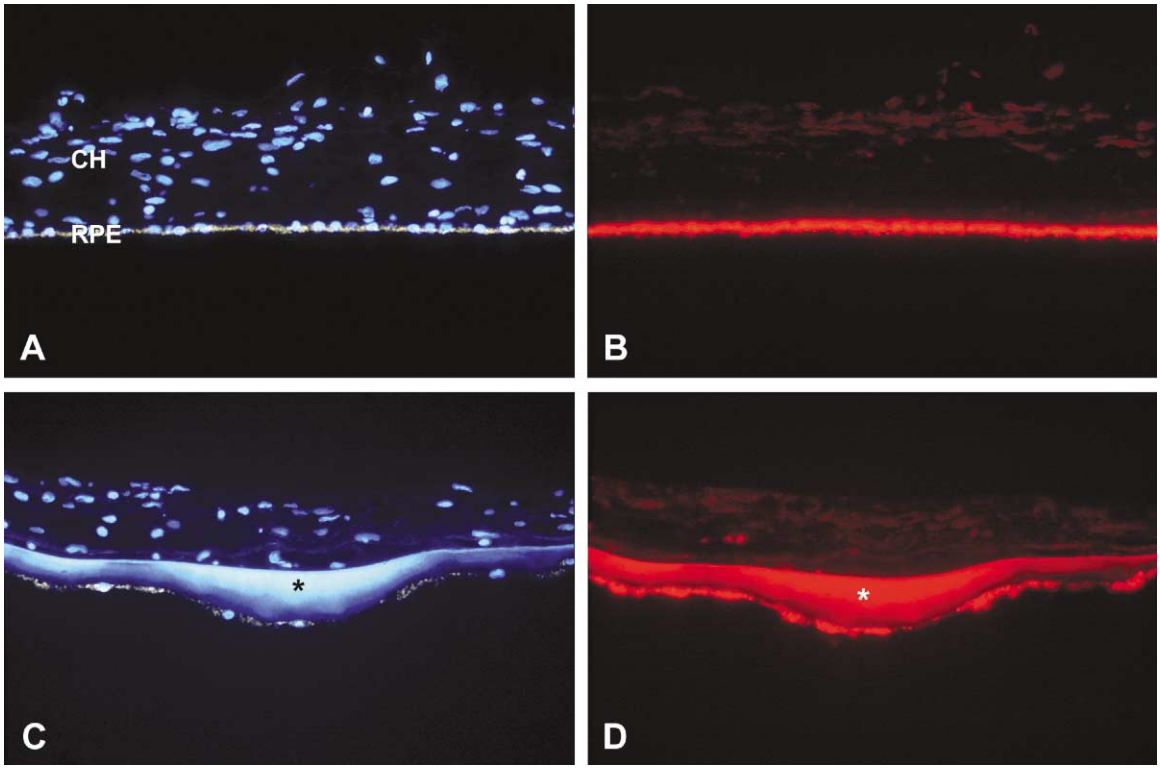

Fig. 9. Light micrographs depicting binding of DAPI, a nuclear stain (A and C), and corresponding autofluorescent images (B and D) are shown. Sections were taken from the central retinas of donor eyes from a 72 year old with no drusen (panels A and B) and a 78 year old with extensive "soft" drusen (panels C and D; asterisks). Note the differences in the density of RPE cell nuclei between these two donors (approximately 21 cells/field (A) vs. approximately 6 cells/field (B)). The drusen are autofluorescent under both wavelengths. CH, choroid; RPE, retinal pigmented epithelium.

et al., 1997). Autoantibodies have been detected in the sera of AMD patients (Guerne *et al.*, 1991; Penfold *et al.*, 1990). Some of these antibodies are directed against specific drusen, RPE and retina components (Hageman and colleagues, unpublished observation).

Moreover, recent immunohistochemical analyses of drusen composition have revealed a distinct array of molecules—including vitronectin, amyloid A, amyloid P component, C5 and C5b-9 terminal complexes, HLA-DR, fibrinogen, Factor X, prothrombin, and in some instances, immunoglobulin—that are common to virtually all clinically defined phenotypes of hard and soft drusen (Hageman and Mullins, 1999; Hageman *et al.*, 1999; Mullins *et al.*, 2000a; Johnson *et al.*, 2000). As discussed above, a number of these constituents (many of which have been thought to be synthesized primarily in the liver) are synthesized locally by RPE, retinal, and/or choroidal cells. These include complement components 5 and 9, immunoglobulin lambda and kappa light chains, Factor X, HLA-DR, apolipoprotein E, amyloid A, and vitronectin.

Notably, a number of drusen-associated constituents are active participants in humoral and cellular immune, and/or inflammatory, processes. Taken together, it is reasonable to propose that immune-related processes may be important in drusen development and the etiology AMD. In evaluating the mechanism(s) of drusen formation, it is indeed difficult to explain the presence of some drusen-associated molecules, especially the terminal complement complex C5b-9, immunoglobulin, and MHC class II antigens, without invoking immune-mediated events. For example, C5b-9

complex formation is associated with specific immune processes and cell death. Thus, the presence of C5b-9, in addition to the presence of vitronectin and other inhibitors of complement mediated cell lysis, in drusen brings into focus the potential role of local complement activation and complement-mediated cell death in drusen biogenesis and the etiology AMD. In this same context, it is interesting to note that drusen are associated with glomerulonephritis, a group of diseases characterized by complement deposition within renal glomeruli. In a recent study involving the eyes from two donors with distinct forms of glomerulonephritis, it was found that Bruch's membrane within these donors possesses numerous drusen that are compositionally similar to the drusen associated with AMD (Mullins *et al.*, 2001). These data suggest that complement activation may play a role in development, and/or that aberrant complement activation or deposition may be sufficient, in some cases, to promote drusen formation. Additional implications of complement activation and its consequences in the aging eye are discussed below.

The co-localization of both immunoglobulin and C5b-9 in drusen also brings to mind the potential role of immune complex formation in the biology of drusen formation. Further analyses will be required in order to determine if the process of drusen formation is a manifestation of immune complex pathogenesis.

Finally, the identification of HLA-DR, a cell membrane-associated protein complex, in drusen was significant in that it suggested the presence of cells and/or cell debris in drusen. This observation, when combined with the identification of novel core domains in drusen (see below), led to the observation that dendritic cells are intimately associated with the process of drusen biogenesis.

4.3. Dendritic cells and drusen biogenesis

As described above, recent histochemical studies have revealed that drusen possess "core" domains which are comprised largely of glycoproteins with O-glycosidically-linked carbohydrate moieties. We have proposed that these localized subdomains represent an early stage in the ontogeny of drusen (Mullins and Hageman, 1999). Further characterization of these cores indicate that they sometimes include bulbous cell processes that breach Bruch's membrane, and terminate as bulbous, vesicle-filled "cores" within the centers of drusen. Ultrastructurally, these processes can be traced back to cell bodies on the choroidal side of Bruch's membrane. These cells immunoreact with a characteristic subset of "cluster differentiation" (CD) antigens and MHC class II antibodies, indicating that they are of monocytic origin. Specific markers including CD1a, CD83, and CD86 antibodies, react with these drusen-associated cells, providing strong evidence that they are dendritic cells that belong to the DC1 lineage (Mullins *et al.*, 2000b). DC1 cells are powerful antigen-presenting cells that are believed to participate in the induction of immunity. These cores are observed in all drusen phenotypes (Hageman and Mullins, 1999), and are present in both macular and extramacular drusen. They are also more prevalent in drusen possessing a height-width ratio of less than 0.5 (Fig. 10). Morphometric analyses indicate that approximately 40% of drusen contain dendritic cell-associated "cores".

4.4. Integrated hypothesis considering the role of inflammation and other immune-mediated processes in drusen biogenesis

We propose herein a unifying hypothesis of drusen biogenesis that attempts to incorporate a large body of new and previously published structural, histochemical, and molecular data pertaining to drusen and drusen composition. This theory is put forth with the acknowledgment that numerous AMD genotypes may exist. Thus, only some aspects of the proposed hypothesis may be involved in any given AMD genotype. This observation invokes, for the first time, the potential for a direct role of cell- and immune-mediated processes in drusen biogenesis. Furthermore, this hypothesis attempts to incorporate new information pertaining to the composition of drusen. Importantly, the theory is based upon novel data documenting that dendritic cells are associated with developing drusen (Fig. 11).

The presence of dendritic cells in inflammatory lesions is well-recognized (Matyszak and Perry, 1997). It is clear that dendritic cells must be

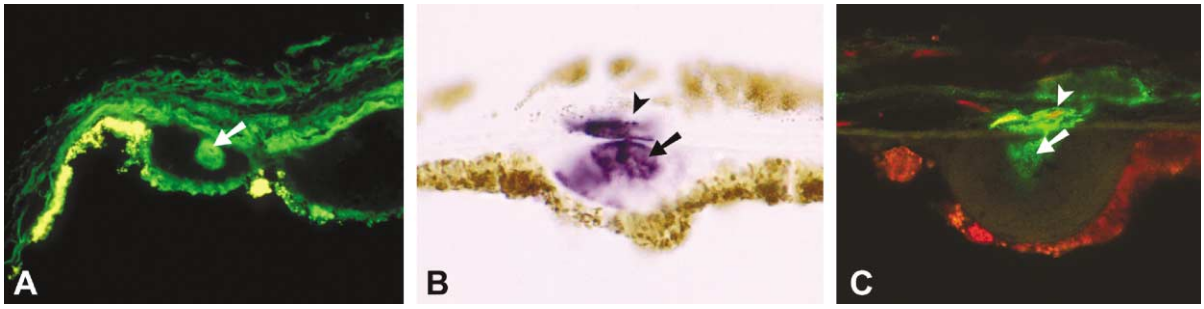

Fig. 10. Light micrographs depicting drusen cores. A single, centralized subdomain bound by PNA lectin following neuraminidase treatment is shown in panel A (arrow). The section in panel B is immunolabeled with antibodies directed against HLA-DR and depicts a strong reaction product associated with both the core (arrow) and a portion of the connected cell body on the choroidal side of the elastic lamina (arrowhead). Weaker HLA-DR antibody-binding material is also present in the druse in a more dispersed pattern. Reactivity of CD68 to the body of a choroidal cell (arrowhead) and its associated drusen core (arrow) is depicted in panel C. The cell process breaching Bruch's membrane is also clearly visible.

recruited, activated, and directed to migrate to, sites of cell and/or tissue "injury". Typically, dendritic cells are recruited by various chemokines, cytokines, heat shock proteins, DNA fragments, complement activation byproducts, and/or other mediators, typically mediated via specific dendritic cell-associated receptors. Choroidal dendritic cell processes are associated with the smallest of drusen, in regions where they have extended a cellular process through Bruch's membrane in order to gain access to the site of tissue damage. They are often observed in the sub-RPE space in association with whole, or portions of, RPE cells that have been shunted into Bruch's membrane, prior to the time that drusen, *per se*, are detectable. Based on these observations and the fact that the RPE is the cell type nearest these dendritic cell processes, we propose that choroidal dendritic cells are "activated and recruited" by locally damaged and/or sublethally injured RPE cells. This idea is consistent with data showing that dendritic cells, and thus the innate immune system, can be activated by microenvironmental tissue damage (Ibrahim *et al.*, 1995; Matyszak and Perry, 1996). Evoking a similar role, we suggest that choroidal dendritic cells serve as sentinel receptors with the capacity to respond to local cell injury, and ultimately provide for the overall integration of immune-mediated processes that determine the outcome of the overall response. It remains to be determined whether drusen-associated dendritic cells initiate a classical immune response involving T helper cells, secrete cytokines that modulate adjacent RPE and/or choroidal cells, elicit an inflammatory or complement-mediated response, or play some other role in the generation of the druse.

In our model, the injured RPE serves as the most likely source of soluble cytokines or other stimulatory factors that initiate dendritic cell recruitment and activation. Collective morphological, biochemical, and molecular data clearly demonstrate that accelerated RPE cell death occurs in eyes derived from donors with AMD, as compared to age-matched controls (see above). Based on available information from other systems, and upon previous suggestions pertaining to the etiology of AMD, RPE cell dysfunction and, ultimately, death might be induced by one or more of several mechanisms, many of which have been proposed previously. These include ischemia, gene-mediated injury, Bruch's membrane-induced dysfunction, oxidative injury from light or systemic factors (e.g. smoking-generated compounds), lipofuscin accumulation, and/or autoimmune-associated processes. It is interesting to note that cells undergoing apoptotic cell death do not typically recruit leukocytes, including dendritic cells. Indeed, based upon data that have been collected thus far, it appears that RPE cell death is most likely due to necrosis, rather than to apoptosis. Ultrastructural and histochemical data provide strong support for necrosis, rather than apoptosis, as the principal pathway leading to RPE cell death.

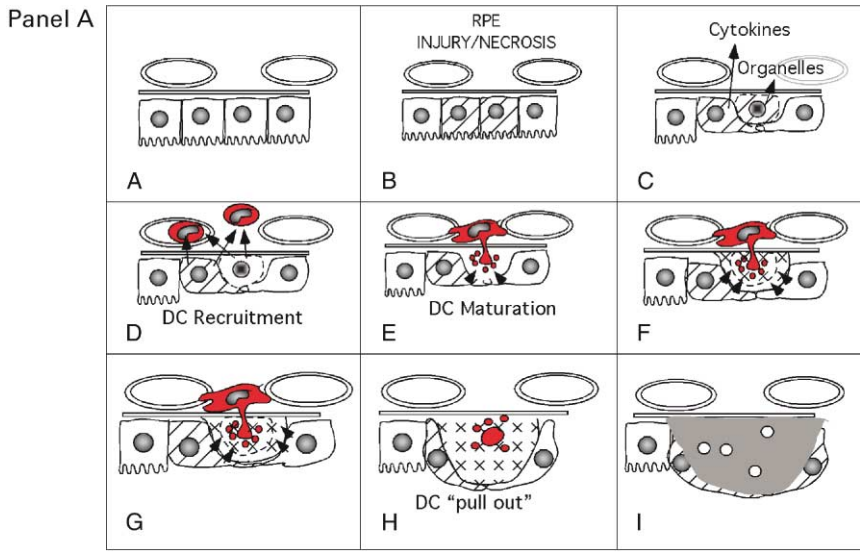

Fig. 11. Schematic depicting the proposed role of inflammatory-, immune-, and cell-mediated events in drusen biogenesis. Panel A depicts the RPE, Bruch's membrane and choroid in the normal condition. In panel B, injury to the RPE occurs through one of any number of proposed mechanisms, including gene mutations, light damage, oxidative stress, lipofuscin accumulation, complement mediated cell injury, etc. This damage results in the release of cytokines and/or RPE "debris" into Bruch's membrane, some of which diffuses into the choroid (C). The RPE cells adjacent to necrotic and/or severely dysfunctional RPE cells form a seal over the RPE debris and synthesize a novel basal lamina, thus maintaining the RPE component of the blood-retinal barrier. Soluble molecules released by the injured RPE likely serve as chemoattractants for choroidal or blood-born monocytes (D), which migrate to the site of injury (the "lesion"), send cellular processes through Bruch's membrane and into the sub-RPE space, and become mature dendritic cells. The bulbous terminations of these processes constitute the drusen "core" (E). A synthetic response by the underlying RPE (F), perhaps in attempts to sequester pathways activated by the dendritic cells, which contributes to the growth of the druse (F, G). Following maturation, the choroidal dendritic cells appear to withdraw their drusen-associated processes, and migrate away from the lesion, leaving behind a residual core of HLA-DR positive material (H), perhaps derived from exosomal secretion, which may disperse during subsequent drusen growth and softening of drusen(I).

Several established pathways can initiate receptor-ligand interactions between dendritic cell precursors and injured tissue, thereby inducing dendritic cell migration and maturation (Ibrahim *et al.*, 1995). A number of these cytokines are synthesized by the RPE, at least in vitro (recently reviewed by Holtkamp *et al.* (2001)). Another recent report indicates that the expression of monocyte chemotactic protein-1 is upregulated in injured ARPE-19 cells (Zheng *et al.*, 2000), providing support for the notion that injured RPE cells may induce monocyte/DC migration. Free radicals, which are known to be present in high concentrations at the RPE-retina-choroid interface (Anderson *et al.*, 1994; Rao, 1990), might be immunostimulatory for dendritic cells. This could explain the general contention that oxidative stress and/or lipofuscin may lead to RPE dysfunction and the development of AMD (Mainster, 1987). Oxidation of proteins and/or lipids at the RPE-Bruch's membrane interface might also participate, either directly or indirectly, in the recruitment of dendritic cells in a fashion similar to that which occurs in atherosclerosis (Glass and Witztum, 2001; Ross, 1999).

Dendritic cells could sustain and amplify the local inflammatory cycle by any number of mechanisms, including immune complex formation, complement activation, extracellular matrix proteolysis, and/or activation of choroidal T-cells or other, phagocytic cells. The presence of numerous immune-associated constituents in drusen, including immunoglobulins, complement proteins, and some acute phase proteins, could be

explained by their involvement in one or more of these events. In addition, the finding that HLA-DR molecules appear to be shed into drusen or the sub-RPE space may suggest that dendritic cells themselves contribute to drusen mass (see Mullins *et al.*, 2000a; Russell *et al.*, 2000), likely through a process of exosome secretion (Thery *et al.*, 1999).

One might predict that the dendritic cell response would be down-regulated once the local tissue damage has been repaired, thus restoring tolerance. This type of self-limiting control is typically accomplished in other systems by migration away from the lesion, attrition or natural killer cell-mediated DC turnover. In the context of drusen biogenesis and AMD, we suggest that a state of local, chronic inflammation at the RPE-Bruch's membrane interface probably persists for many years, never allowing the system to return to tolerance. This might occur as a result of genetic "preprogramming", as in the case of a RPE gene mutation. Local activation of complement, inflammatory cytokines and/or other immunostimulatory events may lead to further focal RPE cell dysfunction, thereby reinforcing a state of "chronic inflammation".

Activation of dendritic cells by local tissue injury might also initiate an autoimmune response to retinal and/or RPE antigens that are uncovered during tissue damage, or via neoantigens that are created within the lesion. Autoimmune responses have been documented in a number of disease processes including, for example, atherosclerosis and ischemia or injury to the heart (Ibrahim *et al.*, 1995). The availability and amount of RPE debris/antigen will most likely determine which ensuing pathway is involved. We note that a number of investigators have identified putative anti-retinal (Guerne *et al.*, 1991; Penfold *et al.*, 1990) and anti-RPE (Hageman and colleagues, unpublished observations) autoantibodies in the sera of individuals with AMD. This might occur as a consequence of aberrant delayed-type hypersensitivity responses, perhaps explaining the presence of serum autoantibodies in at least some AMD patients. It is also conceivable that the groundwork for this process is primed earlier in life by necrosis of RPE cells, potentially explaining the consequence of the wave of peripheral RPE cell dropout we have observed in the second and third decades of life in preliminary studies.

We propose, therefore, that drusen development occurs in at least two distinct stages: a nucleation stage, in which RPE debris and dendritic cell-derived material accumulates in the sub-RPE space, and a maturation stage, in which drusen-associated constituents are deposited around the core. The addition of RPE cell "debris" or proteins secreted in response to the presence of dendritic cells would add to the drusen mass. This type of mechanism would account for the compositional differences between the core and surrounding material.

In the growth or 'maturation' phase, the initiating RPE "lesion" is characterized by the continued deposition of drusen-associated constituents. Early complexes within Bruch's membrane, such as immune complexes, or other local ligands might serve as "nucleation sites" for the deposition of additional self-aggregating proteins and/or lipids. These constituents could be derived from either the plasma and/or local cellular sources. Based on the knowledge that many drusen-associated molecules are circulating plasma proteins, it is plausible that some drusen-associated molecules pass out of choroidal vessels and into the extracellular space adjacent to the RPE where they bind to one or more ligands associated with developing drusen. These ligands could be basement membrane components, plasma membrane receptors, secretory products derived from RPE or choroidal cells, or byproducts of cellular autolysis. We have recently documented the synthesis of a number of drusen-associated molecules, including apolipoprotein E, vitronectin, fibrinogen, C reactive protein, complement 5 and transthyretin, by the RPE and/or retina (Anderson *et al.*, 1999, Anderson *et al.*, 2001; Hageman *et al.*, 1999; Mullins *et al.*, 2000a). Although unexpected, these data support the concept that some drusen-associated may be synthesized and secreted locally. Similarly, the secretion of complement inhibitors, such as vitronectin, in response to local complement activation would contribute to drusen mass. Such a mechanism would also explain why the material within the drusen core is different than more peripheral material and that only one "core" is observed in any given druse

(Mullins and Hageman, 1999). It remains to be determined whether differential regulation of the synthesis of drusen-associated molecules by local cells correlates with drusen deposition and/or AMD.

One might also predict from this model that an imbalance in extracellular matrix synthesis, degradation, and/or turnover could result. In many organs, fibrogenesis is a common complication of tissue injury, independent of the initial site of said injury. In other disease processes, the recruitment of immune cells, and their activation and/or modulation by resident cells, represents a key step in the cascade of events that ultimately lead to fibrosis and/or matrix degradation. One adverse consequence of dendritic cell-mediated drusen biogenesis may be that Bruch's membrane and the surrounding extracellular matrix are degraded by matrix proteases, resulting in a local upregulation of angiogenic factors and opportunistic neovascularization of the sub-RPE and subretinal spaces. Interestingly, dendritic cells do express some proteases which could promote matrix breakdown, including the adamalysins (Wei *et al*., 2001). Moreover, a role for myeloid cells in late stages of AMD has been suggested previously (Killingsworth *et al*., 1990), although there is now considerable evidence for a role of these cells in the early stages of drusen formation.

As the RPE degenerates and drusen become larger and more numerous, there is a concomitant degeneration of photoreceptor cells which may lead to focal serous detachment and gliotic changes in the inner retina. The ensuing cascade of degenerative events eventually manifests itself in the decline of central vision that is characteristic in individuals with AMD.

Based upon data reviewed and presented herein, we hope that other investigators will join us in testing further the various aspects of the propose unifying hypothesis of drusen biogenesis, specifically focusing on the roles of drusen-associated dendritic cells, and associated immune-related pathways. In order to develop a more thorough understanding of the role of immune-mediated processes in drusen biogenesis and the etiology of AMD, we propose that the following issues related to our working hypothesis should be considered in the design of future investigations:

- Are different immune-mediated and/or inflammatory pathways involved in different phenotypes and/or genotypes of AMD?
- Are specific immune-mediated pathways common to all AMD phenotypes and/or genotypes and can these pathways be targeted and modulated "pharmaceutically"?
- What molecular events trigger RPE cell dysfunction in AMD?
- By what mechanism(s) do RPE cells die—apoptosis and/or necrosis—in aging and AMD?
- Does RPE cell loss vary significantly between individuals with and without drusen and/or AMD?
- What relationships exist between RPE cell death, dendritic cells, and other pathologies associated with AMD and drusen formation?
- What precise molecular profiles attract choroidal dendritic cell processes into the sub-RPE space?
- What is the exact role of dendritic cells in drusen biogenesis and the etiology of AMD? Is this role primary or secondary or both?
- Are chronic inflammatory and/or autoimmune phenomena involved in drusen biogenesis and the etiology of AMD?
- Are humoral and/or cellular immune responses involved in drusen biogenesis and early AMD?
- Are immune complexes present in drusen? If so, are the associated immunoglobulins directed against specific local antigens or newly created neoantigens?
- Are specific HLA genotypes associated with specific AMD genotypes and/or phenotypes?
- What is the temporal relationship between complement activation, DC recruitment, chronic inflammation, and drusen biogenesis?

5. SUMMARY AND FUTURE DIRECTIONS

Multiple, independent lines of investigation implicate a role for local inflammatory and immune-mediated events in the development of drusen and, by extension, in the etiology of AMD. These findings are particularly relevant in light of

recent evidence that inflammation- and immune-associated pathways play significant roles in other degenerative diseases associated with advancing age (Akiyama *et al.*, 2000; Glass and Witztum, 2001; Parums *et al.*, 1990; Ross, 1999). In Alzheimer's disease and atherosclerosis, for example, inflammatory "stimuli" (damaged cells, insoluble amyloid deposits) are discrete, localized and present throughout most of life, resulting in the generation of inflammatory processes that are also localized and chronic. Not unlike what seems to be the case in AMD and drusen development, multiple inflammatory subsystems (e.g. complement, cytokine/chemokine, acute phase, coagulation/fibrinolysis, and autoimmune) are involved in these diseases, each with its own amplifying and dampening loops, and multiple interactions with each other. Unraveling the temporal relationships between these various pathways should provide significant insight into the biological mechanisms that mediate drusen biogenesis and the etiology of AMD.

The integrated hypothesis presented herein clearly represents a paradigm shift in our conceptualization pertaining to pathways that participate in the development of drusen and age-related macular degeneration. As with all hypotheses, this one must be rigorously tested. It continues to be our sincere belief, however, that characterization of the earliest cellular and molecular events involved in drusen biogenesis will ultimately lead to novel methods—or the application of current methods—to prevent, delay or slow the progression of this devastating condition.

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To date, over 2000 families have made the difficult decision to donate the eyes of their loved ones, many of whom had poor vision due to AMD, to our research program. It is for these donors and their families, and for the high standard they have set, that we will continue to labor in eager anticipation that treatments for AMD will be forthcoming.

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