The Proteomics of Drusen

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The formation of extracellular deposits known as drusen below the macular region of the retina correlates with increased risk of severe visual loss from age-related macular degeneration (AMD). Inflammation and complement dysregulation contribute to AMD progression; however, disease mechanisms remain incompletely defined. Multiple genetic and environmental factors influence AMD pathology, and although immune system processes play a central role, multiple molecular mechanisms appear to be involved. Drusen proteomics, including the analyses of constituent proteins, oxidative protein modifications, and pattern recognition receptors, provide a foundation for deciphering mechanisms of drusen biogenesis and AMD pathology.

Drusen are extracellular deposits of debris that accumulate with age on Bruch's membrane below the retinal pigment epithelium (RPE) and are considered clinical hallmarks of age-related macular degeneration (AMD). AMD, the leading cause of blindness in the elderly worldwide, is a complex disease involving inflammation and, in part, dysregulation of the complement system (Jager et al. 2008; Ding et al. 2009; Anderson et al. 2010). To date, 19 genetic loci (Fritsche et al. 2013) and multiple environmental factors, including diet and smoking, have been associated with AMD risk (Jager et al. 2008; Ding et al. 2009). Clinicians refer to drusen as either "soft" or "hard" in describing their relative shape and size, with hard drusen being nodular and generally smaller than soft drusen, which have a more diffuse appearance (Hageman et al. 2001). The presence of numerous and/or confluent soft drusen in the macula is considered a major risk factor for develop-

ment of advanced AMD with severe visual loss (Fig. 1). Only 10%–20% of cases of early/mid-stage AMD (also known as "dry" AMD) progress to advanced AMD, with neovascular or "wet" AMD (characterized by choroidal neovascularization) being the more prevalent form of advanced AMD (Ferris et al. 1984; Zarbin 2004). Geographic atrophy, also known as advanced dry AMD, is characterized by focal atrophy of the RPE and loss of macular photoreceptors, whereas choroidal neovascularization involves abnormal blood vessel growth from the choriocapillaris through the RPE, resulting in possible hemorrhage, exudation, scarring, and/or retinal detachment. It is possible for the two forms of advanced AMD to occur at the same time in separate eyes of an individual. Antivascular endothelial growth factor (VEGF) treatments can slow the progression of choroidal neovascularization (Jager et al. 2008), but universally effective therapies for geographic at-

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Figure 1. Comparison of ocular tissues containing drusen from normal eyes and eyes from AMD patients. In the normal retina (A), the photoreceptor cells (P) are highly organized, and their light-sensitive outer segments (white arrows) are in close contact with the RPE. Bruch's membrane (dark arrows), a thin layer of extracellular matrix between the RPE and choriocapillaris (CH), is barely visible at this magnification. In the AMD retina (B), photoreceptors are highly disorganized because of the presence of drusen (D), which also distorts the RPE and Bruch's membrane. (From Bok 2002; reproduced, with permission from National Academy of Sciences USA © 2002.)

rophy or for the prevention of wet or dry AMD have yet to be developed.

Drusen are widely accepted as contributors to the progression of AMD but the molecular mechanics of drusen formation are not yet well defined. Many components of drusen have been identified however the details of drusen biogenesis continue to be sought for molecular insights into AMD prevention and therapy. Histochemical and immunohistochemical studies have shown that drusen contain a variety of lipids, polysaccharides, glycosaminoglycans and proteins (Abdelsalam et al. 1999; Hageman et al. 1999, 2001; Kamei and Hollyfield 1999; Mullins et al. 2000; Malek et al. 2003; Anderson et al. 2004; Curcio et al. 2005a,b; Luibl et al. 2006; Wang et al. 2010; Jiang et al. 2012). Proteomic studies, both qualitative and quantitative, have expanded the repertoire of proteins and protein modifications associated with drusen (Crabb et al. 2002a; Umeda et al. 2005; Yuan et al. 2010; Garland et al. 2013). This review focuses on the proteomic components of drusen, and their potential impacts on drusen biogenesis and AMD pathology.

QUALITATIVE PROTEOMIC STUDIES

By the year 2000, about 20 protein components of human drusen had been identified by immunohistochemistry, including several immune

system proteins, leading to the hypothesis that drusen formation involves inflammatory and immune responses to RPE damage (Hageman et al. 1999; Johnson et al. 2000; Mullins et al. 2000). In 2002, unbiased proteomic analysis by liquid chromatography tandem mass spectrometry (LC MS/MS) identified 129 proteins from isolated human drusen (Crabb et al. 2002a). Drusen are often found in the retinal periphery of normal aged eyes, and this early qualitative proteomic analysis included drusen isolated from 18 normal donors and 5 AMD donors. Based on the frequency of detection, common proteins found in both AMD drusen and normal donor drusen included tissue inhibitor of metalloproteinase 3 (TIMP3), clusterin, vitronectin, serum albumin, and crystallins, with crystallins detected more frequently in AMD donor drusen than in normal donor drusen. Significant proteomic heterogeneity was also apparent, with about a third of the proteins detected in AMD drusen not observed in normal donor drusen. To date, this study remains the only reported LC MS/MS proteomic comparison of human drusen purified from AMD eyes and normal eyes, in part because of the difficulty in obtaining AMD specimens and to the laborious drusen isolation procedure (Crabb et al. 2002a). Another mass spectrometric study has identified 60 proteins from drusen isolated from cynomolgus monkeys (*Macaca fascicula*-

ris) exhibiting macular degeneration (Umeda et al. 2005). About 50% of the monkey proteins were identical to or homologous with human drusen components. The currently identified proteins from isolated primate drusen constitute a subset of the drusen proteome, as many other proteins could be present as shown by the quantitative proteomic studies discussed below.

Immunocytochemical evidence has corroborated the drusen localization of several of the proteins identified by LC MS/MS, including annexins I and VI, proteins S100 A7, S100 A8, and S100 A9 (Crabb et al. 2002a), clusterin (Sakaguchi et al. 2002), TIMP3 (Kamei and Hollyfield 1999), vitronectin (Hageman et al. 1999), amyloid A, amyloid P, α 1-antitrypsin, α 1-antichymotrypsin, apolipoprotein A1, apolipoprotein E (APOE), multiple complement components (C3, C5, and C9), fibrinogen, immunoglobulin κ , immunoglobulin λ , and ubiquitin (Mullins et al. 2000). Annexin II, identified by LC MS/MS, was localized to the basal plasma membrane of the RPE adhering to drusen (Crabb et al. 2002a). Among the primate drusen proteins identified by LC MS/MS, it is possible that others may also belong to the RPE or Bruch's membrane. Nevertheless, this proteome supports the idea that drusen proteins come from several sources including the RPE, choroidal vasculature (Hageman et al. 2001; Penfold et al. 2001), and the systemic circulation.

QUANTITATIVE PROTEOMIC STUDIES

Two quantitative proteomic studies provide additional insights into mechanisms of drusen formation and macular degeneration. These studies include an analysis of the macular region of the Bruch's membrane/choroid complex from human AMD donors and normal donors (Yuan et al. 2010), and a proteomic analysis of Bruch's membrane/choroid from a mouse model of Doyme honeycomb retinal dystrophy/malattia Leventinese (Garland et al. 2013). Bruch's membrane is an important extracellular, semi-permeable support for the RPE, influences the diffusion of nutrients and waste products between the RPE and the choroidal bloodstream, and restricts cell migration (Goldberg 1976). It

is composed of the basement membranes of the RPE and choriocapillaris, inner and outer collagenous layers, and a central elastin zone (Booij et al. 2010). AMD pathology typically involves thickening and decreased permeability of Bruch's membrane (Goldberg 1976), thought to be caused in part by accumulation of collagen, lipids, advanced glycation endproducts (AGEs), RPE waste products and cross-links that covalently trap debris (Booij et al. 2010).

AMD-Associated Protein Alterations in Human Macular Bruch's Membrane/Choroid

LC MS/MS isobaric tags for relative and absolute quantitation (iTRAQ) technology has been used to quantify proteins in the macular region of the Bruch's membrane/choroid complex from human postmortem eyes (10 with early/mid dry AMD, six with advanced dry AMD, eight with wet AMD, and 25 normal eyes) (Yuan et al. 2010). A total of 901 proteins was quantified, the majority of which differed little in amount between AMD and controls and thus provide a partial proteome of normal human macular tissues of average age 81. Ninety-nine proteins were identified with significantly altered amounts in AMD macular tissues relative to normal controls, including 56 elevated and 43 reduced proteins. Consistent with proposed mechanisms of Bruch's membrane thickening (Booij et al. 2010), the relative amounts of 13 different collagens and protein-glutamine γ -glutamyltransferase 2, a cross-linking enzyme, were elevated in AMD tissues (Yuan et al. 2010). About 60% of the elevated proteins were immune response and host defense proteins, including many complement proteins and complement-associated proteins (e.g., C3, C4A, C5, C6, C7, C8 γ , C9, complement factor B, complement factor H (CFH), and clusterin). Also among the elevated proteins were several damage-associated molecular pattern proteins (DAMPs) such as α -defensins 1-3, protein S100-A8, protein S100-A9, α -crystallin A, α -crystallin B, histones and galectin-3. DAMPs are endogenous proteins released by damaged cells and capable of activating pattern recognition receptors (Lotze et al. 2007). Not surpris-

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ingly, several proteins identified in isolated drusen were elevated in the macular AMD tissues (Table 1).

Inflammatory response proteins were found to be elevated in multiple categories of AMD progression, supporting the idea that inflammatory processes are involved in both initiating events of AMD and progression to advanced AMD. Four retinoid processing proteins (retinal pigment epithelium-specific 65 kDa protein, cellular retinoic acid-binding protein 1, cellular retinaldehyde binding protein, and interphotoreceptor retinoid binding protein) were found uniquely elevated in early/mid stage AMD, suggesting that retinoid metabolism contributes to AMD initiation, likely through mechanisms involving RPE lipofuscin (Eldred and Lasky 1993; Sakai et al. 1996; Sparrow and Boulton 2005; Kim et al. 2007; Sparrow 2007; Ng et al. 2008; Wu et al. 2010). AGE receptor 3 (also known as galectin-3) was the most significantly elevated protein in advanced dry AMD, implicating a role for AGEs in geographic atrophy. The remaining ~40% of the elevated proteins were associated with other regulatory functions and specialized metabolic or housekeeping processes. Elevated regulatory proteins included the protease inhibitors TIMP3 and α -1-microglobulin as well as pigment epithelium-derived factor, a neurotrophic antiangiogenic protein, all of which were also detected in purified drusen (Crabb et al. 2002a). Other elevated regulatory proteins in macular Bruch's/choroid included phosphatidylethanol-amine binding protein 1, a serine protease inhibitor; Rho GDP-dissociation inhibitor 1, a cell adhesion regulator; reticulon-4, a cell migration regulator; and voltage-dependent anion-selective channel protein 3, a diffusion regulator of small molecules. Consistent with proposed roles for mitochondrial dysfunction in aging (Knott et al. 2008) and AMD pathology (Nordgaard et al. 2008), mitochondrial cytochrome c oxidase subunit 5B, 2-oxoglutarate/malate carrier protein, and mitochondrial aldehyde dehydrogenase were elevated in the macular AMD Bruch's membrane/choroid complex in AMD.

Significantly reduced proteins accounted for a small fraction (<2%) of the proteins quan-

tified in the macular region of the Bruch's/choroid complex in AMD. Over half of these proteins can be associated with cell adhesion and protein interactions (asporin, nidogen-2, Protein FAM 10A4, CD9), or vascularization and angiogenesis (tryptase α 1, plasmalemma vesicle associated protein, caveolin-1, β 2-glycoprotein 1). Proteins decreased only in early/mid stage AMD implicate hematologic malfunctions, reduced extracellular matrix integrity, and weakened cellular interactions. Proteins uniquely decreased in advanced dry AMD included β -crystallin B2, carbonic anhydrase 4, ubiquitin, and haptoglobin. Neuroblast differentiation-associated protein, a membrane remodeling and repair protein also known as desmoyokin, was decreased in advanced dry AMD but elevated in neovascular AMD. Proteins uniquely reduced in neovascular AMD included two histones, CD 9, three glycolytic enzymes, mitochondrial trifunctional enzyme β , creatine kinase B and ferritin light chain. Interphotoreceptor retinoid binding protein was reduced in both advanced dry and wet AMD. While reduced protein abundance in advanced AMD could reflect disease consequences (e.g., cell death), the majority of elevated proteins were different in geographic atrophy compared with neovascular AMD, implicating multiple mechanisms of AMD progression.

Proteins in the Bruch's Membrane/Choroid in *Efemp1*^{R345W/R345W} Mice

Doyne honeycomb retinal dystrophy/malattia Leventinese is an inherited macular dystrophy caused by a point mutation (R345W) in the gene that encodes epidermal growth-factor-containing fibulin-like extracellular matrix protein 1 (EFEMP1), yielding a human phenotype similar to dry AMD with extensive drusen (Stone et al. 1999, 2004; Marmorstein 2002; Michaelides et al. 2006; Fu et al. 2007). Gene-targeted *Efemp1*^{R345W/R345W} mice provide an animal model that with age exhibit a macular degeneration-like phenotype and extensive formation of extracellular basal laminar deposits under the RPE (Fu et al. 2007; Marmorstein et al. 2007). Such basal deposits have long been con-

Table 1 Drusen proteins found in elevated amounts in macular Bruch's membrane/choroid of AMD eyes

SwissProt accession	Protein	Subcellular source	All AMD			Early/mid stage AMD			Advanced dry AMD			Advanced wet AMD		
			Mean protein ratio	Donors total =	P value	Mean protein ratio	Donors total =	P value	Mean protein ratio	Donors total =	P value	Mean protein ratio	Donors total =	P value
P05109	Protein S100-A8	Cytoplasmic	1.7	12	<0.01	1.6	5	<0.01				1.7	4	0.02
P07360	Complement C8γ	Secreted	1.7	7	0.04									
P13671	Complement C6	Secreted	1.6	4	0.03									
P10643	Complement C7	Secreted	1.6	8	<0.01	2.0	3	0.03						
P02763	α1-acid glycoprotein 1	Secreted	1.5	9	<0.01	1.5	4	<0.01				1.6	3	0.03
P06702	Protein S100-A9	Cytoplasmic	1.4	12	<0.01				1.7	3	0.02	1.9	4	0.01
P35625	TIMP3	Secreted	1.4	24	<0.01	1.4	10	<0.01	1.3	6	0.02	1.4	8	<0.01
P02489	α-Crystallin A	Cytoplasmic	1.3	13	<0.01	1.6	6	0.05						
P02760	AM BP protein	Secreted	1.3	3	0.04									
P01031	Complement C5	Secreted	1.3	10	<0.01	1.5	5	<0.01						
P06899	Histone H2B type 1-J	Nuclear	1.2	9	<0.01	1.2	3	0.02	1.2	3	0.04			
P04004	Vitronectin	Secreted	1.2	24	<0.01							1.3	8	<0.01
P36955	Pigment epithelium-derived factor	Secreted	1.2	8	0.01									
P02748	Complement C9	Secreted	1.2	24	0.01	1.2	10	0.03				1.5	8	0.01
P10909	Clusterin	Secreted	1.1	24	<0.01				1.2	6	0.04			
P02511	α-Crystallin B	Cytoplasmic	1.1	24	<0.01	1.3	10	<0.01						
P01011	α1-antichymotrypsin	Secreted	1.1	24	0.07	1.2	10	0.05				1.2	8	0.05
P01024	Complement C3	Secreted	1.1	22	0.02							1.2	7	0.01

Proteins were identified in isolated human drusen (Crabb et al. 2002a) and quantified in separate analysis of human macular Bruch's membrane/choroid from 24 AMD donors and 25 normal control donors (Yuan et al. 2010).

Mean protein ratios (AMD/control), donor frequency and p values (t-test) are shown. Proteins were considered significantly elevated if quantified in ≥ 3 donor specimens per group with protein ratios > 1 standard deviation above the group mean protein ratio and with P values ≤ 0.05 .

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sidered potential precursors of drusen (Sarks 1976; Green and Enger 1993; Spraul and Grossniklaus 1997; Curcio and Millican 1999; Sarks et al. 2007).

Quantitative proteomic analysis of basal deposits in the *Efemp1*^{R345W/R345W} mouse model has revealed important clues to mechanisms of basal deposit formation (Garland et al. 2013). Bruch's membrane/choroid specimens from three ages of mutant and control mice (8, 14, and 24 mo) were analyzed to detect protein changes from early through extensive basal deposit formation. Bruch's membrane specimens without RPE or choroid were analyzed at 24 mo to more closely measure the basal deposit proteome. About 780 proteins per animal were identified, yielding a total of 1062 proteins quantified using LC MS/MS spectral counting methods. Major changes in protein abundance in the mutant mice were found associated with immune system processes. Notably, 11 complement components were detected in the samples from *Efemp1*^{R345W/R345W} mice, with significant increases relative to controls in the abundance of complement components C3 and C4 in both 24-mo Bruch's membrane samples and in Bruch's/choroid specimens by 14 mo. The functional role of the complement system in basal deposit formation was further tested by generating homozygous *Efemp1*^{R345W/R345W};C3^{-/-} double mutant mice that expressed the R345W point mutation but lacked C3 expression. Histological analyses showed that basal deposits were markedly reduced in the double mutant mice, demonstrating that an active complement system plays an important role in basal deposit formation in this mouse model. The molecular details of this process, however, remain to be determined.

Quantitative proteomic changes in *Efemp1*^{R345W/R345W} mutant mice highlighted three other major biological processes that potentially contribute to basal deposit formation, namely cell–cell and cell–matrix adhesion, signal transduction, and intracellular transport (Garland et al. 2013). Four proteins exhibiting large increases in the Bruch's membrane of mutant mice and of possible pathological significance were EFEMP1, thrombospondin 1, milk-

fat globule-EGF factor 8, and collagen VI, each of which can be associated with multiple processes and/or extracellular matrix structure function. No new or unusual proteins were identified, suggesting that normal extracellular matrix components in altered amounts or structure contribute to basal deposit formation. Similarities in the proteomic results from Bruch's membrane/choroid from *Efemp1*^{R345W/R345W} mice (Garland et al. 2013) and human AMD donors (Yuan et al. 2010) were significant. About 50% of the proteins identified in the human study were also detected in the mouse samples, with remarkable quantitative agreement between select proteins, including C3, C4, vitronectin, galectin-3, and Ig μ chain C region, which were increased in both advanced AMD patients and the mutant mice.

OXIDATIVE PROTEIN MODIFICATIONS IN DRUSEN

Oxidative stress has long been considered a major contributor to AMD pathology (Seddon et al. 1996; Beatty et al. 2000; AREDS 2001; Hageman et al. 2001). Because of high oxygen tension and light exposure, the RPE/Bruch's/choroid interface is an extreme environment that facilitates the production of reactive oxygen species and nitrogen species that can stimulate inflammatory and immune responses (Verhaselt et al. 1998; Matsue et al. 2003; Hazen 2008; Hagenow et al. 2009). Moreover, in this extreme environment, photoreceptor outer segment tips phagocytized daily by the RPE are highly susceptible to oxidation, resulting in the production of reactive cleavage fragments from lipids, sugars, and retinoids, and subsequent oxidative modifications. A host of elevated protein modifications derived from lipoxidation and glyco-oxidation have been associated with AMD ocular tissues (Crabb 2012), and several have been demonstrated in drusen.

Oxidative protein modifications have the potential to mask proteolytic cleavage sites, inactivate enzymes (Crabb et al. 2002b), and contribute to the accumulation of cross-linked intracellular debris that copurifies with RPE lipofuscin granules (Chio et al. 1969; Schutt et al.

2003; Ng et al. 2008) and with extracellular debris in drusen (Crabb et al. 2002a). For example, several drusen proteins have been shown to migrate in multiple mass ranges in SDS gel electrophoresis, from the top to the bottom of the gel (Crabb et al. 2002a). The higher-mass components containing TIMP3, vitronectin, and C9 suggest the presence of covalent cross-links caused by reactive oxidation fragments from lipids and/or carbohydrates (Friguet et al. 1994; Elgawish et al. 1996).

Carboxymethyllysine was the first oxidative protein modification reported in drusen (Fig. 2). Greater immunoreactivity for carboxymethyllysine was detected in basal laminar and basal linear deposits, soft macular drusen, and choroidal neovascular membranes from AMD eyes compared to control eyes (Ishibashi et al. 1998). Carboxymethyllysine is a lysine glycoxidation modification formed through the non-enzymatic Maillard reaction, which combines sugar carbonyls with primary amino groups to form Amadori products. Amadori products un-

dergo subsequent nonenzymatic reactions, including oxidative decomposition, to form a heterogeneous group of modifications known as AGEs (Baynes 2001). The presence of AGEs in aging ocular tissues is well-established (Ishibashi et al. 1998; Farboud et al. 1999; Hammes et al. 1999; Handa et al. 1999; Crabb et al. 2002a; Howes et al. 2004; Glenn et al. 2007, 2009). Pentosidine, another AGE in drusen, forms fluorescent lysine–arginine cross-links (Handa et al. 1999; Glenn et al. 2007). Advanced glycation occurs slowly over time, and long-lived proteins (e.g., collagens) in extracellular matrices such as Bruch's membrane are particularly susceptible to modification. AGE modifications promote protein denaturation, decreased solubility, decreased flexibility, and increased resistance to proteolytic degradation. Several studies have demonstrated that carboxymethyllysine induces VEGF expression in vitro in a variety of cell types, (Hirata et al. 1997; Lu et al. 1998; Treins et al. 2001; Hoffmann et al. 2002; Urata et al. 2002) and stimulates neovascularization in vivo

Figure 2. Oxidative Protein Modifications in Human Drusen and Bruch's Membrane. (A) Immunohistochemical analysis of Bruch's membrane/choroid tissue with anticarboxyethylpyrrole monoclonal antibody. (A') Control section, probed with preimmune IgG. (Arrows indicate Bruch's membrane and drusen; scale bar, 50 μ m). (B) Immunohistochemical analysis with anticarboxymethyllysine monoclonal antibody. (B') Control consecutive section treated with normal mouse IgG. This figure demonstrates the lipoxidation product carboxyethylpyrrole and the glycoxidation product carboxymethyllysine associated with drusen and Bruch's membrane. (Scale bar, 50 μ m). (From Crabb et al. 2002a; reproduced, with permission from National Academy of Sciences USA © 2002.)

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(Okamoto et al. 2002). Plasma levels of both carboxymethyllysine and pentosidine are elevated in AMD (Ni et al. 2009).

Several receptors modulate the biological effects of AGEs (Goh and Cooper 2008), including receptor of AGEs (RAGE), a pattern-recognition receptor that binds a variety of ligands, including DAMPs. RAGE ligands vary in their proinflammatory potential, but carboxymethyllysine is a strong inducer of RAGE signaling (Herold et al. 2007). Drusen proteins S100A8 and S100A9 (Crabb et al. 2002a; Yuan et al. 2010) are DAMPs thought to be activators of RAGE (Sparvero et al. 2009). Treatment of cultured RPE cells with AGEs, including carboxymethyllysine, has been shown to upregulate RAGE, resulting in apoptotic RPE cell death (Howes et al. 2004).

Multiple lipoxidation products are present in drusen, Bruch's membrane, and the cellular debris that copurifies with RPE lipofuscin granules (Crabb et al. 2002a; Gu et al. 2003a; Schutt et al. 2003; Shen et al. 2007; Ng et al. 2008; Weismann et al. 2011; Shaw et al. 2012). Lipoxidation in drusen is not surprising given the high amounts of cholesterol esters and phospholipids in drusen (Wang et al. 2010). Reactive lipid fragments can form Schiff base linkages with primary amino groups, resulting in the formation of a variety of adducts (Esterbauer et al. 1991; Gu et al. 2003b). Carboxyethylpyrrole was the first lipoxidation product identified in drusen (Fig. 2) and has been shown to be significantly more abundant in AMD ocular tissues and blood than in normal tissues (Crabb et al. 2002a; Gu et al. 2009). Carboxyethylpyrrole adducts are generated by the covalent reaction of primary amino groups (e.g., protein ϵ -lysyl NH_2) with 4-hydroxy-7-oxohept-5-enoic acid, a reactive fragment derived uniquely from oxidative cleavage of docosahexaenoate (DHA)-containing lipids (Gu et al. 2003b). DHA is abundant in the retina (Fliesler and Anderson 1983) and concentrated in the photoreceptor outer segments and the RPE (Wang and Anderson 1992; Alvarez et al. 1994). Mass spectrometric analysis of carboxyethylpyrrole-immunoreactive 2D gel spots has identified albumin and α 1-antitrypsin as likely carboxyethylpyrrole-

modified proteins in the Bruch's membrane/choroid/RPE complex in AMD; both of these proteins have been found in drusen (Crabb et al. 2002a).

Carboxyethylpyrrole adducts stimulate angiogenesis *in vivo* through toll-like receptor 2 (TLR2), another pattern recognition receptor. In contrast to neovascularization induced by carboxymethyllysine, carboxyethylpyrrole stimulates angiogenesis independent of VEGF (Ebrahim et al. 2006; West et al. 2010). Similar to complement and RAGE, TLR2 binds multiple ligands and initiates inflammatory and immune responses (Zahringer et al. 2008). At low concentrations, carboxyethylpyrrole adducts are hypothesized to contribute to wound healing, but at high concentrations may catalyze amplified TLR2 signaling and excessive neovascularization (West et al. 2010). Carboxyethylpyrrole protein adducts have also been shown to promote platelet activation and aggregation *in vitro* and thrombosis *in vivo* through toll-like receptor 9 (TLR9) (Panigrahi et al. 2013). Mice immunized with carboxyethylpyrrole-adducted mouse albumin develop focal changes in the RPE resembling those in advanced dry AMD, exhibit monocyte and macrophage migration into the interphotoreceptor matrix and also exhibit elevated complement deposition in Bruch's membrane (Hollyfield et al. 2008). Carboxyethylpyrrole adducts appear to be initiators of immune responses capable of contributing to either dry or wet AMD-like phenotypes.

Other lipoxidation products in drusen appear to be ligands for CFH, including malondialdehyde (Weismann et al. 2011) and 1-palmitoyl-2-(5'-oxo-valeroyl)-sn-glycero-3-phosphocholine (POVPC) (Shaw et al. 2012). CFH exhibiting the AMD risk 402H polymorphism was found to bind malondialdehyde or POVPC with less affinity than the CFH 402Y non-risk variant (Weismann et al. 2011; Shaw et al. 2012). These independent studies also reported that inflammatory responses stimulated by malondialdehyde and POVPC *in vitro* and *in vivo* were stronger with the AMD risk variant of CFH than with the nonrisk CFH isoform, suggesting that the nonrisk isoform may help dampen inflammatory responses induced by oxidative

modifications. These observations add support to the hypothesized central role of oxidative modifications in the pathology of AMD and justify further mechanistic studies.

DRUSEN FORMATION

Immune-mediated events have long been thought to play a role in drusen formation due to the presence of proteins in drusen upregulated during inflammatory responses (Johnson et al. 2000; Hageman et al. 2001). Growing evidence supports this hypothesis. Consistent with early drusen studies, several genes involved in inflammation, and/or the immune system have now been associated with AMD risk. These include CFH (Edwards et al. 2005; Hageman et al. 2005; Haines et al. 2005; Klein et al. 2005), complement factor B (Gold et al. 2006), complement C3 (Yates et al. 2007), APOE (Zarepari et al. 2004; Baird et al. 2006), toll-like receptor 4 (Zarepari et al. 2005), LOC387715/ARMS2 (Rivera et al. 2005; Kanda et al. 2007), HTRA1 (Dewan et al. 2006; Yang et al. 2006), and ABCA4 (Allikmets 2000). In a human RPE cell culture model mimicking early AMD, drusen biogenesis was reported to involve select serum protein interactions with APOE-rich sub-RPE deposits, which subsequently activate the complement cascade and the deposition of complement complexes (Johnson et al. 2011). Amyloid β (A β) may also play a role in drusen formation (Johnson 2002; Dentchev et al. 2003; Anderson et al. 2004; Luibl et al. 2006; Isas et al. 2010). Notably, drusen-like deposits develop below the RPE following A β treatment of mice lacking the A β -degrading enzyme neprilysin (Yoshida et al. 2005). In the human *APOE4-knockin* mouse model of AMD, A β is found in sub-RPE deposits and neovascular lesions (Malek et al. 2005; Ding et al. 2008), and anti-A β therapy blocks histopathologic changes (Ding et al. 2011). In human drusen, A β has been colocalized with activated complement (Johnson 2002; Dentchev et al. 2003; Anderson et al. 2004). A β has also been reported to activate complement (Rogers et al. 1992; Jiang et al. 1994) and other pattern recognition receptors, including RAGE (Chen et al. 2007; Schmidt

et al. 2009) and toll-like receptors (Fassbender et al. 2004; Liu et al. 2005). As recently reported and described above, an active complement system has an important function in basal deposit formation in *Efemp1*^{R345W/R345W} mutant mice (Garland et al. 2013). While specific molecular mechanisms remain to be defined, immune system processes appear to play a major role in drusen formation.

CONCLUDING REMARKS

Drusen, when concentrated on Bruch's membrane in the macular region, are major risk factors for AMD progression to advanced disease. Early immunohistochemical studies suggested that inflammatory and immune response processes after RPE injury contribute to drusen formation, a hypothesis that has gained significant support. Qualitative proteomic analysis of isolated drusen using mass spectrometry has made important contributions toward understanding drusen formation, including the identification of multiple immune responses and cellular defense proteins, and support for multiple sources of drusen components, including the RPE, choroidal vasculature, and the systemic circulation. Quantitative proteomic analysis of the human Bruch's membrane/choroid complex has identified 99 proteins significantly altered in abundance in the macular region of AMD tissues, many of which are involved in immune system and cellular defense processes, and also were previously identified in isolated human drusen. Quantitative proteomics and genomics have also demonstrated a critical role for the complement system in basal deposit formation in a mouse model of Doyme honeycomb retinal dystrophy/malattia Leventinese, with remarkable quantitative agreement between select proteins from human and mouse Bruch's membrane/choroid complex. Double mutant mice expressing the disease-causing variant of EFEMP1 protein but not expressing C3 exhibited markedly reduced basal deposit formation, demonstrating that in this mouse model, the immune system plays a major role in basal deposit formation.

Immune system processes involving pattern recognition receptors other than the comple-

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ment system may also contribute to AMD pathology. Several studies have shown that drusen and ocular tissues from AMD donors contain a variety of oxidative protein modifications, including lipoxidation products (e.g., carboxyethylpyrrole, malondialdehyde, and POVPC), glycoxidation products (e.g., carboxymethyllysine and pentosidine), and protein cross-links. Whereas CFH has been reported to bind malondialdehyde and POVPC, oxidative modifications such as carboxymethyllysine and carboxyethylpyrrole trigger immune responses through other pattern recognition receptors such as RAGE, TLR2, and TLR9. Drusen and the macular Bruch's membrane/choroid complex from AMD donors contain elevated levels of several DAMPS (e.g., protein S100s, crystallins, and histones) that may also trigger pattern recognition receptors. Other proteins such as A β and the cross-linking enzyme protein-glutamine γ -glutamyltransferase 2 also may have important roles in drusen formation. Multiple molecular mechanisms appear to be involved and more work is required to fully understand drusen biogenesis.

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