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Catherine Bowes Rickman
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Retinal Degenerative Diseases

Mechanisms and Experimental Therapy

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Preface

The International Symposia on Retinal Degeneration have been held in conjunction with the biennial meeting of the International Society for Eye Research (ISER) since 1984. These RD symposia have allowed basic and clinician scientists from around the world to convene and present their new research findings. They were organized to allow substantial time for discussions and one-on-one interactions in a relaxed atmosphere, where international friendships and collaborations could be fostered. The 18th International Symposium on Retinal Degeneration (also known as RD2018) was held from September 3 to 8, 2018, in the marvelous Victorian Hotel, The Great Southern, in the beautiful city of Killarney, Ireland. The meeting brought together 286 basic and clinician scientists, retinal specialists in ophthalmology, and trainees in the field from all parts of the world.

Abstract submissions to the RD2018 meeting exceeded all expectations, both in quantity and quality. The scientific program covered many aspects of retinal degeneration. The presentations included 44 platform talks and 153 posters. The program consisted of 3 full days of platform talks and 2 evening poster sessions. The RD2018 meeting was highlighted by four special keynote lectures. The first keynote lecture was given by *James Handa, MD*, Johns Hopkins University, who discussed "The RPE in AMD: Are They on an Inevitable Journey to Death?". *Rando Allikmets, PhD*, of Columbia University gave the second keynote lecture titled "Solving Stargardt/ABCA4 Disease by Integrating Clinical and Genetic Analyses." *Jacque Duncan, MD,* University of California at San Francisco, presented the third keynote lecture titled "Retinal Structure and Function in Patients with Retinal Degenerations." The fourth and final keynote lecture was given by *Peter Humphries, PhD*, Trinity College Dublin, who discussed "On Experimental Approaches to Molecular Therapy for Retinal Degeneration." The scientific meeting ended with a "Welcome to RD2020" by Local Organizer Juan Gallo, MD, along with the organizers primarily responsible for the meeting, Drs. John D. Ash and Eric Pierce.

We thank the Local Organizing Committee Chairs, *Dr. Peter and Marian Humphries*, Trinity College Dublin, and their Local Organizing Committee Members, Drs. *Laura Brady*, Fighting Blindness Ireland; *Matthew Campbell* and *Jane Farrar*, Trinity College Dublin; and *Paul Kenna*, Research Foundation, Royal Victoria Eye and Ear Hospital Dublin. In addition, we thank the outstanding management and staff of the Great Southern Hotel and Conference Center for their assistance in making this an exceptionally

smooth-running conference and a truly memorable experience for all of the attendees. These included, in particular, *Denise O'Sullivan* and *Aine McMahon*.

We were very pleased to be able to fund 65 "full-ride" travel awards for graduate students, postdocs, and junior faculty, the largest number of travel awards for an RD meeting to date! Travel awards were made possible in part by funding from the National Eye Institute (NEI) of the National Institutes of Health. We are pleased to report this is the ninth consecutive symposium in which the NEI has contributed travel awards to support young investigators. We are also grateful to the Foundation Fighting Blindness (FFB) for their continuous support since 1986. FFB, then known as the National Retinitis Pigmentosa Foundation, started supporting our meeting from our second meeting, RDII, and we dedicated our first volume (RDI) to Ben Berman who was the founder of FFB. Additional awards were provided by generous national and international financial support from a number of organizations, including the BrightFocus Foundation (since 2014), Pro Retina Germany (1998 and since 2012), the Fritz Tobler Foundation Switzerland (since 2012), Bayer, Novartis/Fighting Blindness Ireland, Science Foundation Ireland, Fáilte Ireland, and Biosciences Ireland. Many of the contributing foundations sent members of their organizations to attend the meeting. Their participation and comments in the scientific sessions were instructive to many, offering new perspectives to some of the problems being discussed.

We also acknowledge the diligent and outstanding efforts of Ms. *Holly Whiteside* and *Jazzamine Asberry*. Holly recently retired as the Administrative Manager of Dr. Anderson's laboratory at the University of Oklahoma Health Sciences Center. She had been the RD Symposium Coordinator since 2000. Her duties were taken over for the RD2018 meeting by Jazzamine.

Finally, we acknowledge the contributions since 1984 of Matthew LaVail, one of the three founding organizers of the RD meetings. Matt has retired from the University of California at San Francisco and will no longer contribute (officially) to the ongoing RD meetings.

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Part V Inherited Retinal Degenerations

Part I

Age-Related Macular Degeneration (AMD)

AMD-Associated HTRA1 Variants Do Not Influence TGF-β Signaling in Microglia

1

Isha Akhtar-Schaefer, Raphael Reuten, Manuel Koch, Markus Pietsch, and Thomas Langmann

Abstract

Genetic variants of high-temperature requirement A serine peptidase 1 (HTRA1) and age-related maculopathy susceptibility 2 (ARMS2) are associated with age-related macular degeneration (AMD). One HTRA1 single nucleotide polymorphism (SNP) is situated in the promotor region (rs11200638) resulting in increased expression, while two synonymous SNPs are located in exon 1 (rs1049331:C > T, rs2293870:G > T). HtrA1 is known to inhibit transforming growth factor-β (TGF-β) signaling, a pathway regulating quiescence of microglia, the resident immune cells of the brain and retina. Microglia-mediated immune responses contribute to AMD pathogenesis. It is currently unclear whether AMD-associated HTRA1 variants influence TGF-β signaling and microglia phenotypes. Here, we show that an HtrA1 isoform carrying AMD-associated SNPs in exon 1 exhibits increased proteolytic activity. However, when incubating TGF-β-treated reactive microglia with HtrA1 protein variants, neither the wildtype nor the SNP-associated isoforms changed microglia activation in vitro.

Keywords

Age-related macular degeneration (AMD) · Genome wide association studies (GWAS) · Single nucleotide polymorphisms (SNPs) · AMRS2/HTRA1 · Transforming growth factor β (TGF-β) · TGF-β signaling · Microglia · Microglial quiescence · Immunomodulation

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1.1 Introduction

Genome-wide association studies (GWAS) identified strong correlations between polymorphisms on chromosome 10q26 and AMD (Yang et al. 2006).

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This locus harbors the genes for the HTRA1/ARMS2. The risk haplotype contains several SNPs, with one of which situated in the promoter region of HTRA1 (rs11200638), resulting in increased transcript and protein levels (Tuo et al. 2008). HtrA1 was found in drusen and the aqueous humor of AMD patients (Tosi et al. 2017). Mice overexpressing HtrA1 exhibited Bruch's membrane (BM) fragmentation which is characteristic for AMD (Vierkotten et al. 2011; Nakayama et al. 2014).

TGF-β is a well-defined regulator of immune regulation (Goumans et al. 2009). In the retina TGF-β belongs to factors released by the RPE into the subretinal space and exhibits immunosuppressive functions on the innate immune system by modulating microglia (Zamiri et al. 2007). Microglia, the resident immune cells in the retina, are responsible for tissue repair and pro-inflammatory responses associated with disease progression (Akhtar-Schafer et al. 2018). TGF-β potently attenuates neuroinflammation by dampening microgliosis (Zöller et al. 2018). Whether HtrA1 contributes to inflammation by inhibiting TGF-β signaling remains largely elusive. An AMD-associated HtrA1isoform, which carries two synonymous SNPs in exon 1 (rs1049331 and rs2293870), exhibited reduced binding to TGF-β in microglia (Friedrich et al. 2015). In the present study, we asked whether HtrA1 triggers pro-inflammatory microglia responses by interfering with TGF-β signaling.

1.2 Methods

1.2.1 Eukaryotic Protein Expression

The ORFs of wildtype (WT) and protease inactive (S328A) mouse (m) HtrA1 and WT, S328A, and AMD-associated (SNP) human (h) HtrA1 variants were cloned into an expression plasmid containing an N-terminal 2x Strep II tag. The S328A mutants contained a substitution of serine at position 328 with alanine, while the SNP isoform carried two SNPs in exon 1 (rs1049331:C > T: A34; rs2293870:G > T: G36). Proteins were expressed as previously described (Reuten et al. 2016).

1.2.2 Protease Assay

Enzymatic activity of HtrA1 was assessed using the EnzChek Protease Assay Kit (Thermo Fischer Scientific). HtrA1 proteins diluted in cold digestion buffer (10 mM Tris-HCL pH 7.8, 0.1 mM NaN_3) were pipetted into a black 96-well microtiter plate. Reaction was initiated by adding 5 μg/ml BODIPY-FL casein. Increasing concentrations of HtrA1 were applied. The increase in relative fluorescence units (RFUs) at excitation/emission wavelengths of 485/535 nm was monitored every 20 seconds at 37 °C using an Infinite®F200 Pro plate reader (Tecan). Values were baseline corrected by subtracting the mean RFU increase without any enzyme.

1.2.3 Cell Culture

BV-2 microglia-like cells were cultured as previously described (Scholz et al. 2015). Cells were treated with 10 ng/ml interferon-γ (IFN-γ) or 1 ng/ml TGF-β (PreproTech) or both for 24 h. Treated cells were preincubated with 50 ng/ml, 100 ng/ml, and 150 ng/ml HtrA1 protein. For Smad2 expression analysis, cells were incubated with TGF-β and HtrA1 proteins for 3 h.

1.2.4 Quantitative Real-Time RT-PCR

RNA extraction and first-strand cDNA synthesis was performed as described previously (Madeira et al. 2018). Amplifications of 50 ng cDNA were performed with the LightCycler® 480 Instrument II (Roche). For the detection of plasminogen activator inhibitor 1 (Pai-1), iNos and arginase-1 (Arg1) intron spanning primers were used. ATP5B served as reference gene. The reactions were subjected to 40 cycles of amplification (95 °C for 15 s, 60 °C for 1 min). Relative quantification was performed using the LightCycler® 480 software 1.5.1.

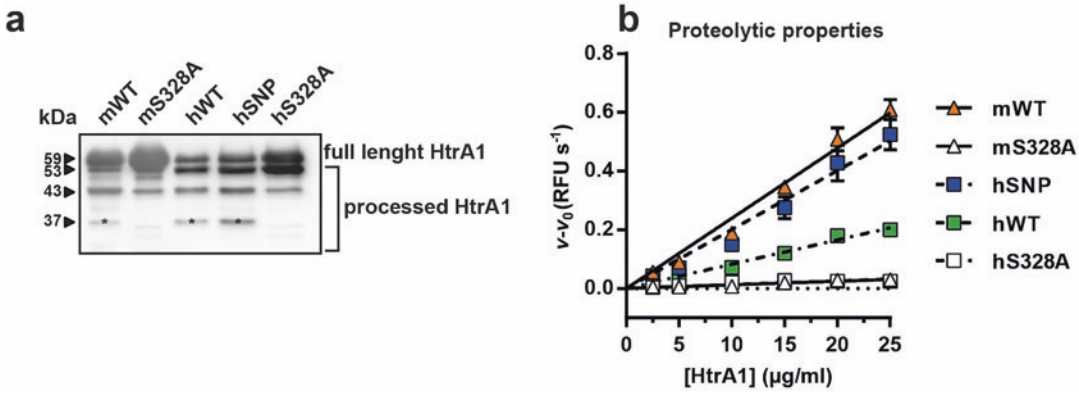

Fig. 1.1 (**a**) Immunoblot of recombinant HtrA1 shows autolytic cleavage and smaller fragments which are absent in mS328A and hS328A (asterisk). (**b**) Slopes of enzymatic rates. Shown are mean values ± SEM ($n = 3$)

1.2.5 Western Blot

Isolated proteins were subjected to Western blot analysis as previously described (Aslanidis et al. 2015). Primary antibodies against pSamd2 and Smad2 (Cell Signaling Technology) and secondary goat anti-rabbit IgG-HRP were used.

1.2.6 Statistical Analysis

Real-time RT-PCR data was analyzed using ANOVA followed by Tukey's multiple comparison test. pSmad2/Smad2 Western blot analysis data was analyzed using the non-parametric Kruskal-Wallis test, followed by Dunn's multiple comparison correction test using GraphPad Prism version 6.07. $P \leq 0.05$ which was considered as statistically significant.

1.3 Results

1.3.1 HtrA1 Protein Variants Are Proteolytically Active

We expressed recombinant mWT, mS328A, hWT, hSNP, and hS328A HtrA1. HtrA1 proteins were detected at approximately 59 kDa (Fig. 1.1a). Smaller fragments were partly attributed to autolytic cleavage. Quantitative protease assay using BODIPY FL-labeled casein demonstrated reproducible proteolytic activity of mWT, hWT, and hSNP forms (Fig. 1.1b).

1.3.2 HtrA1 Does Not Reduce TGF-β Induced Microglial Quiescence

Treatment of BV-2 cells with TGF-β led to a significant mRNA increase in its response gene Pai-1 (Fig. 1.2a). IFN-γ treatment induced a significant upregulation of iNos and reduction of the alternative activation marker Arg1 (Fig. 1.2d, g). TGF-β treatment reduced the pro-inflammatory phenotype of microglia by decreasing iNos and increasing Arg1 transcripts. When mWT was applied, we observed a significant decrease in Pai-1, Arg1, and iNos (Fig. 1.2b, e, h). However, when the human forms were applied, no significant changes were observed in transcript levels of any marker when compared to IFN-γ + TGF-β (Fig. 1.2c, f, i).

1.3.3 HtrA1 Protein Variants Do Not Change TGF-β Induced pSmad2 Levels

Smad2 proteins are phosphorylated upon TGF-β receptor activation. Hence, we evaluated the influence of HtrA1 variants on pSmad2 levels in TGF-β-treated BV-2 cells (Fig. 1.3). Application of 1 ng/ml TGF-β led to an increase in pSmad2 levels. However, co-treatment with any of the HtrA1 variants did not change pSmad2 levels.

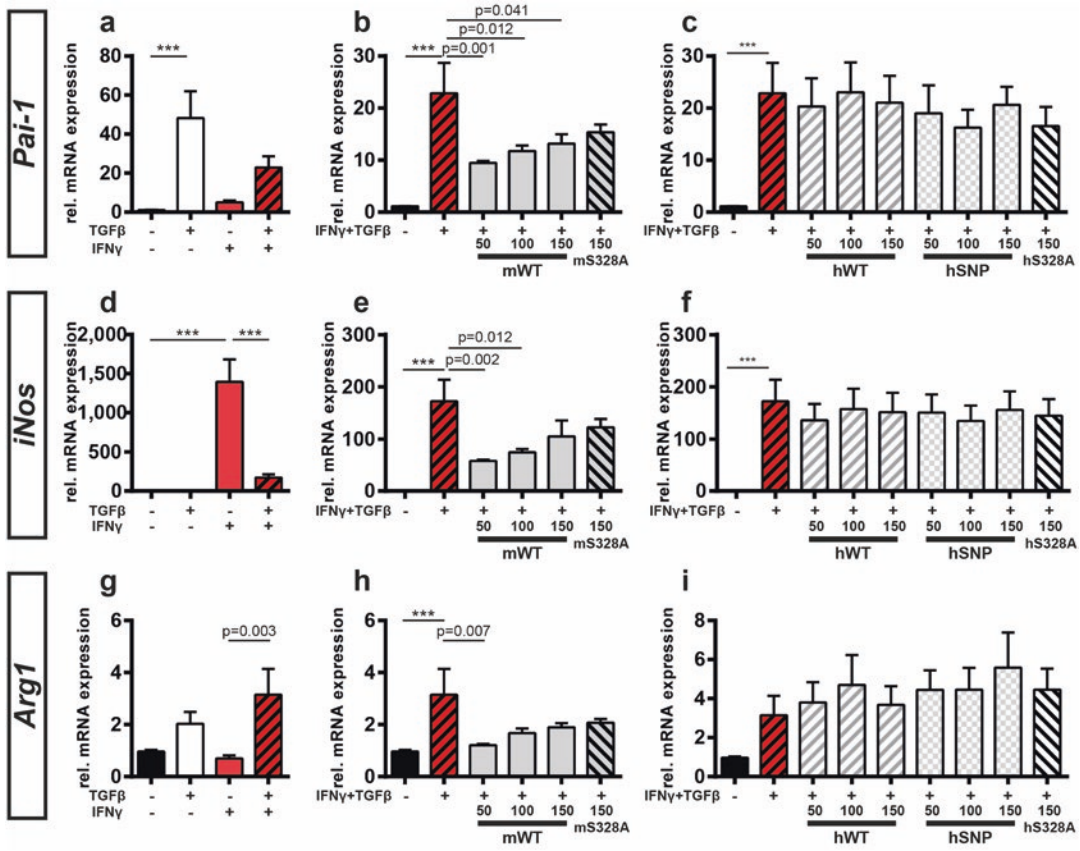

Fig. 1.2 Treatment of BV-2 cells with TGF-β led to a significant increase in Pai-1 (**a**–**c**). Treatment with IFN-γ induced significant upregulation of iNos and reduction in Arg1. Co-treatment with TGF-β reduced the pro-inflammatory phenotype (**d**, **g**). Application of low-dose mWT led to a significant decrease in Pai-1 and Arg1 but also in iNos (**b**, **e**, **h**). Application of human HtrA1 led to no changes (**c**, **f**, **i**). Normalized values are presented as mean ± SEM. ($n = 9$) with $***p \leq 0.001$

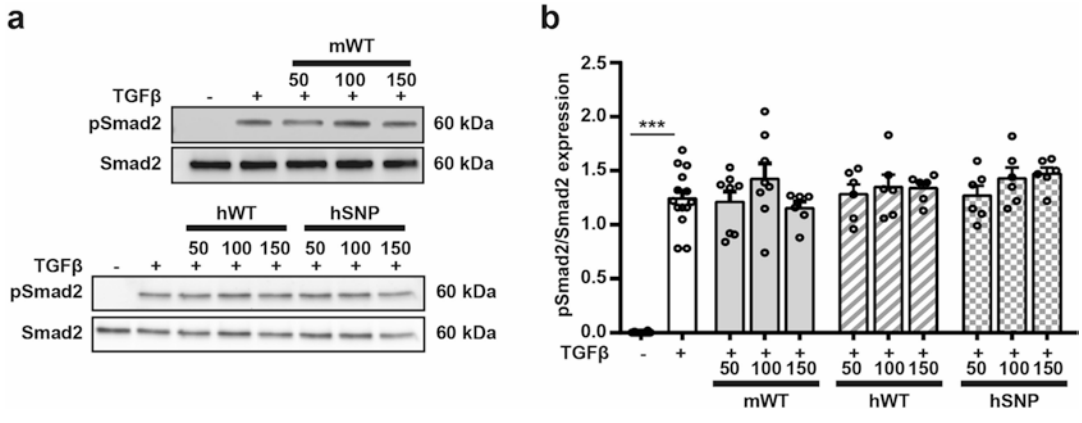

Fig. 1.3 HtrA1 does not change levels of pSmad2 after TGF-β stimulation. (**a**) Densitometric analysis of blots shown in (**a**) (**b**). Values are presented as mean ± SEM. ($n = 6$–14) with $***p \leq 0.001$ when compared to TGF-β stimulation

1.4 Discussion

Here, we evaluated the effect of AMD-associated HtrA1 isoforms on microglia in vitro. First, all HtrA1 proteins, apart from the S328A forms, were proteolytically active. Interestingly, we observed increased catalytic activity of the proteins with the hSNP variant.

Second, we observed that TGF-β significantly inhibited IFN-γ-mediated iNos mRNA production, as previously described (Zöller et al. 2018). However, we did not observe an influence of HtrA1 on microglial TGF-β signaling. Similar findings were reported with mucosal mast cells, where no inhibition of TGF-β signaling by HtrA1 was observed. (Gilicze et al. 2007)

Our results showed no differences between the wild-type and AMD-associated HtrA1 isoforms regarding TGF-β signaling. The synonymous AMD-associated SNPs are located in the insulin-like growth factor (IGF-1)-binding domain, and hSNP variants showed reduced ability to bind IGF-1 and TGF-β (Jacobo et al. 2013; Friedrich et al. 2015). However, these findings are challenged by our results. Further studies will have to address the question whether AMD-associated HtrA1 isoforms may influence retinal microglia in vivo.

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2 A Review of Pathogenic Drivers of Age-Related Macular Degeneration, Beyond Complement, with a Focus on Potential Endpoints for Testing Therapeutic Interventions in Preclinical Studies

Mayur Choudhary and Goldis Malek

Abstract

Age-related macular degeneration (AMD) continues to be the leading cause of visual impairment for the elderly in developed countries. It is a complex, multifactorial, progressive disease with diverse molecular pathways regulating its pathogenesis. One of the cardinal features of the early clinical subtype of AMD is the accumulation of lipid- and protein-rich deposits within Bruch's membrane, called drusen, which can be visualized by fundus imaging. Currently, multiple in vitro and in vivo model systems exist, which can be used to help tease out mechanisms associated with different molecular pathways driving disease initiation and progression. Given the lack of treatments for patients suffering from the dry form of AMD, it is imperative to appreciate the different known morphological endpoints associated with the various pathogenic pathways, in order to derive further insights, for the ultimate purpose of disease modeling and development of effective therapeutic interventions.

Keywords

Age-related macular degeneration · Lipid metabolism · Inflammation · Quick-freeze/deep etch · Retinal pigment epithelial cells · Cholesterol · Apolipoprotein

2.1 Introduction

Age-related macular degeneration (AMD) is a progressive and complex age-related disease. It is the leading cause of vision loss among people over 50 years of age in the Western world. Since the principle risk factor for AMD is advanced age, the number of people afflicted with AMD is estimated to rise to 288 million people by 2040 (Wong et al. 2014). A better understanding of the pathogenesis of AMD is crucial in identification and/or development of preclinical models, ultimately leading to effective therapeutics that may prevent or reverse this disease.

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To date, several classification schemes of AMD have been described, based on in vivo imaging using color fundus photos and optical coherence tomography. The Age-Related Eye Disease Study (AREDS) is one of the most well-known systems of classification (Age-Related Eye Disease Study Research 2000). It classifies AMD into early, intermediate, and late stages. A major clinical feature of the disease is extracellular deposition of lipids and proteins underneath the retinal pigment epithelium (RPE) known as drusen. Early-stage "dry" AMD is characterized by the presence of medium-sized drusen (>63 μm; <125 μm) and pigmentary abnormalities; intermediate-stage "dry" AMD is defined by the presence of at least one large druse (>125 μm) and numerous medium-sized drusen, or RPE atrophy excluding the macular region. Advanced-stage AMD can manifest in two forms that may coexist: (1) geographic atrophy (GA) or late "dry," affecting 85–90% of patients, characterized by several large drusen and RPE atrophy extending to the center of the macula and (2) exudative AMD, affecting 10–15% of patients, defined by choroidal neovascularization and any of its associated sequelae such as subretinal fluid, hemorrhage, RPE detachment, and/or fibrotic scarring (Malek and Lad 2014). A subset of exudative AMD patients respond to antiangiogenic treatment targeting vascular endothelial growth factor (VEGF), whereas the quest for an effective therapy for GA remains elusive to date, due to its diverse and complex pathology, which involves multiple mechanisms including but not limited to dysregulation of lipid metabolism and transport, inflammation, complement pathway dysregulation, extracellular matrix (ECM) remodeling, cell death, and cell adhesion. The focus of this mini review is on the modeling of the pathobiology of dry AMD.

2.2 Pathobiology of AMD

The pathogenesis of AMD is influenced by cross talk between components of the retinal microenvironment, namely, photoreceptors, RPE cells, Bruch's membrane (BrM), choriocapillaris, and the outer choroid. Though complement has been shown to play an important part in regulating the health of the choriocapillaris as well as the RPE (Chirco et al. 2016), several other pathways have been shown to regulate the early stages of AMD including lipid metabolism and transport, inflammation, and ECM remodeling, whereas the late "dry" AMD seems to converge into pathogenic pathways such as cell senescence and death (Miller et al. 2017). Thus it would be valuable to develop a targeted approach to understanding modeling of disease phenotypes as well as identify quantifiable endpoints targeting these pathways.

2.2.1 Lipid Metabolic Dysregulation in AMD

One of the defining characteristics of early "dry" AMD is the accumulation of lipid-rich deposits between the RPE and BrM, as well as within BrM, which vary in size, thickness, and confluence (Klein et al. 1991). It has been shown that at least 40% of drusen volume is comprised of lipids (Wang et al. 2010). Components of drusen are derived from the retina, RPE, and to a lesser extent, the choroidal circulation. This retention of lipids causes formation of a variety of deposits, not only drusen but also basal laminar deposits (BLamD, between RPE and its plasma membrane) and basal linear deposits (BLinD, between the RPE and inner collagenous layer of BrM) (Curcio et al. 2011). Furthermore, genome-wide association studies (GWAS) of AMD patients have led to the identification of multiple lipid metabolism-related genes like *ABCA1*, *ABCA4*, *APOE*, *CETP*, and *LIPC* (Yu et al. 2011; Merle et al. 2013). Complementary to the GWAS reports, several epidemiological studies that have investigated the role of statins and AMD, point toward an association between circulating lipid levels and drusen formation (Klein et al. 2014; Vavvas et al. 2016). Possible mechanisms of statin therapy have been postulated, including changes in lipoprotein metabolism, improvement in lipid efflux, lipid clearing by macrophages, and anti-inflammatory and protective effects on

RPE cells. Collectively, these studies suggest that AMD may be reversible anatomically and functionally, and establishes lipid metabolism and transport as a viable target for disease modeling and therapy development.

Key players in cholesterol transport and lipid metabolism are apolipoproteins (apo), proteins that have been shown to accumulate in drusen. Accordingly, multiple studies have been conducted to date investigating the role of apolipoproteins using in vivo modeling, often incorporating an additional stressor such as dietary manipulation. For example, *apo∗E3*-Leiden mice (modeling human type III hyperlipoproteinemia) when fed a high-fat diet for 9 months developed BLamD, composed of electron dense material similar to that seen in human AMD and immunoreactivity toward apoE, supporting apoE's involvement in BLamD development (Kliffen et al. 2000). Further, aged mice expressing the human *APOE4* allele maintained on a high cholesterol diet developed AMD-like pathology, including diffuse sub-RPE deposits, thickened BrM, and RPE atrophy (Malek et al. 2005). The apolipoprotein (apo) A-I mimetic peptide 4F, a small anti-inflammatory and anti-atherogenic agent, when delivered via intravitreal route in *apoE*^{null} mice displayed improvement in BrM's health and lessened the esterified cholesterol levels in BrM (Rudolf et al. 2018). Since apoB lipoprotein particles have been detected in BrM in early-stage "dry" AMD, transgenic mice expressing human apoB100 have been generated. High-fat diet and photooxidative injury in these transgenic mice resulted in loss of basal infoldings and cytoplasmic vacuoles in the RPE, in addition to accumulation of BLamD and long-spacing collagen in BrM (Espinosa-Heidmann et al. 2004; Fujihara et al. 2009). Additionally mice expressing mouse apoB100 develop lipoprotein accumulations in their BrM (Fujihara et al. 2014). These studies are consistent with the hypothesis that under conditions of hyperlipidemia, the RPE may secrete apoB100-rich lipoproteins to counter lipotoxicity, which may lead to the formation of lipid-rich deposits as seen in early-stage "dry" AMD. This may explain the aging effect, which in combination with photooxidative injury, leads to lipid accumulation, culminating in the formation of sub-RPE deposits. The accumulation of drusen can be exacerbated by inflammation, as discussed in the next section.

2.2.2 Inflammation in AMD

Inflammation and immune dysfunction are other pathogenic mechanisms associated with AMD development and central to early stages of the disease. With aging, the retina suffers from a low-grade chronic oxidative insult, which it reportedly sustains for decades. Therefore, the inflammation/"para-inflammation" may at a given point cross a threshold, become pathogenic, and lead to disease development. Noteworthy is that human histological/biochemical evaluation of para-inflammation in the AMD eye is limited to studies that have shown involvement of CD163^{+}, CD68,$^{+}$ and complement factor-3^{+} cells in AMD specimens (Lad et al. 2015; Wang et al. 2015; Natoli et al. 2017). In light of the "para-inflammation" hypothesis, the potential for modulating the inflammatory response is currently being evaluated as a therapeutic avenue in AMD.

An element of inflammation that has been postulated to regulate AMD pathogenesis is the NACHT, LRR, and PYD domains containing protein 3 (NLRP3) inflammasome. It is a large multiprotein complex within the immune cells that functions in the innate immune response pathway as a molecular platform for activation of caspase-1 and subsequent maturation and secretion of biologically active interleukin-1β (IL-1β) and IL-18 (Schroder and Tschopp 2010). Terallo et al. described that accumulation of Alu RNA transcripts in RPE following DICER1 loss primed and activated the NLRP3 inflammasome in RPE, leading to IL-1β- and IL-18-mediated RPE degeneration (Tarallo et al. 2012).

2.3 Potential Endpoints for Preclinical Studies

This mini review has attempted to present some aspects of dry AMD pathobiology, with a focus on the role of lipid metabolism and inflammation

in the progression of the disease. Transgenic mouse models are an effective tool to study the contribution of specific molecular pathways to the pathogenicity of AMD. Targeting molecular pathways necessitates the use of unambiguous endpoints to elucidate the role of a particular pathway. For example, characterization of lipids present in mouse BrM requires staining reagents to establish the normal baseline, which then may be compared against genetic or dietary manipulations. Oil red O is a histological tool that may be employed to stain a class of lipids called neutral lipids which encompasses triglycerides (TG), esterified cholesterol (EC), and fatty acids (FA). Furthermore, EC and unesterified cholesterol (UC) can be distinguished by a fluorescent, polyene antibiotic filipin stain. Successful filipin staining has been shown in human tissue sections and cultured cells (Curcio et al. 2011). Ultrastructural studies using transmission electron microscopy (TEM) have long been a benchmark to study deposits in human tissue and animal models. It provides detailed visualization of photoreceptor outer segments, RPE cells and BrM. Using TEM, studies have illustrated electron dense materials below the RPE, RPE pigmentary changes, BrM thickening, and changes in the choriocapillaris. Detailed views of ocular lipoprotein particles and ECM changes have also been made possible by quick-freeze/deep etch, a tissue preparation technique used in conjunction with electron microscopy that can produce three-dimensional images of tissue structure and macromolecular elements while preventing the introduction of post-processing artifacts (Ismail et al. 2017). Finally, immunohistochemical stains may be used to visualize lipid accumulations using antibodies against apoB, apoE, perilipin, and E06, which bind to proteins covalently modified by oxidized phospholipids (Malek et al. 2003; Harris et al. 2013).

Assessment of inflammation is also important as an in vitro, ex vivo, or in vivo endpoint and may be performed by surveying for immune cells. Retinal and RPE-choroid flatmounts are an excellent way to get an overview of the resident microglial cells to establish a baseline. The number and density of microglia can be used to evaluate the effect of injury/insult or transgene expression. Various antibodies can be employed for this purpose, namely, Iba1 (ionized calcium-binding adaptor molecule 1), F4/80, CD68, and CD45. In addition to the number/density of immune cells, the morphological characterization of microglia/macrophage may be used as a measure of inflammatory status. Macrophages can differentiate into either classically activated M1 phenotype, characterized by the expression and production of pro-inflammatory mediators including IL-1β, TNF-α, and IL-6 as well as an increased expression of surface markers such as CD16/32, CD86, CD40, and inducible nitric oxide synthase, which have been reported to drive the inflammatory process. Additionally, the M2 macrophages express high levels of arginase-1 and IL-10 but low levels of IL-12 and IL-23 and are usually induced by anti-inflammatory cytokines IL-4 and IL-13.

2.4 Summary

Investigation into the pathological mechanisms of a complex and multifactorial disease such as AMD may entail the use of an assortment of model systems, each of which may target distinct disease-associated pathways. It is imperative to develop a comprehensive understanding of potential endpoints associated with each model system in order to delve deeper into mechanistic questions as well as develop effective therapeutic modalities.

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GPR143 Signaling and Retinal Degeneration

3

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Abstract

Age-related macular degeneration (AMD) is the most common cause of irreversible blindness. We do not know the cause of the disease and have inadequate prevention and treatment strategies for those at risk or affected. The greatest risk factors include age and race, with the white population at the highest risk for the disease. We developed the hypothesis that pigmentation in the retinal pigment epithelium (RPE) protects darkly pigmented individuals from AMD. We have tested this hypothesis in multiple ways including dissecting the pigmentation pathway in RPE using albinism-related tools, identification of a G protein-coupled receptor in the pigmentation pathway that drives expression of trophic factors, and using a very large retrospective chart analysis to test whether the ligand for the receptor prevents AMD. In total, our results indicate that pigmentation of the RPE is a cornerstone of RPE-retinal interaction and support and that the receptor in the pigmentation pathway most likely underlies the racial bias of the disease. The ligand for that receptor is an ideal candidate as a preventative and treatment for AMD. Here we summarize these results, discussing the research in its entirety with one overall goal, treatment or prevention of AMD.

Keywords

AMD · Albinism · Retinal degeneration · Pigmentation · GPCR · GPR143 · OA1 · Dopamine · L-dopa · PEDF

3.1 Introduction

Age-related macular degeneration (AMD) is the most common cause of irreversible visual loss in the developed world, reviewed in (Jager et al. 2008). Despite years of intensive research, the pathogenesis and etiology of AMD are unknown. Some of the identified risk factors for AMD include genetics, age, gender, smoking, sunlight exposure, and race (Ehrlich et al. 2008). AMD is not a genetic disease, but clear association with various risk alleles within the complement system is strongly supported (Hageman et al. 2005; Loane et al. 2011; Holliday et al. 2013; Cascella et al. 2014). The immune system participates in the pathologic process of AMD, but whether that is a response to tissue injury or a primary cause of the disease remains unclear.

Race is one of the strongest risk factors for AMD, with the white population particularly susceptible (Fig. 3.1a). Race is a complex trait based

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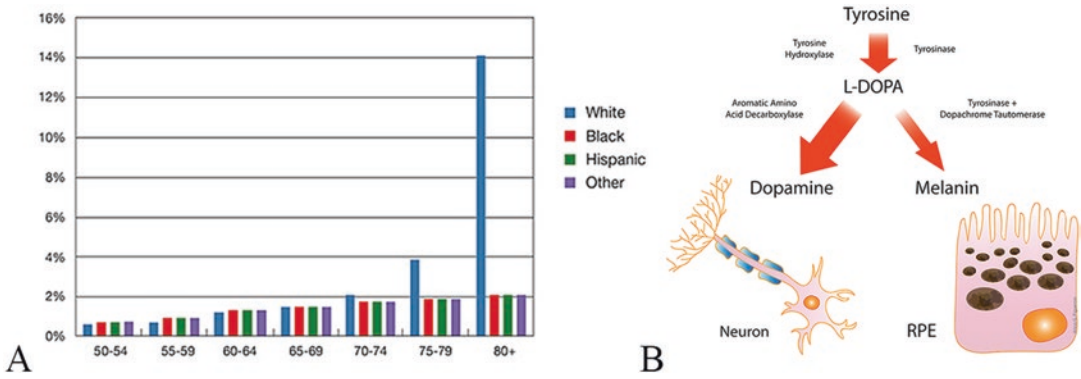

Fig. 3.1 (**a**) Percentage of individuals with intermediate and advanced age-related macular degeneration separated by age and race. Adapted from the National Eye Institute (**b**) Overview of melanin synthesis compared to neuronal dopamine synthesis. Both melanin and dopamine are derived from tyrosine, with an intermediate production of L-dopa

on many genes, but retinal pigment epithelium (RPE) pigmentation may be a key factor for the racial bias of AMD. Lessons from albinism, the loss of pigmentation, illustrate how RPE pigmentation supports the neurosensory retina. All forms of albinism are genetic, and all exhibit the same retinal phenotype, reviewed in (Mckay 2018). Albinism from any cause, be it a lysosomal storage defect, enzymatic dysfunction in melanin synthesis, or loss of a G-protein-coupled receptor (GPCR), each causes a similar retinal phenotype. People with albinism frequently exhibit low vision due to developmental changes in the neurosensory retina, despite the fact that the retina never expresses any of the genes that cause albinism. The degree of visual problems varies among affected individuals, generally along with the residual levels of RPE pigmentation. Finally, the retinal consequences of albinism are the same in all races directly indicating that the relationship between RPE pigmentation and retinal support transcends the other genetic traits involved in race. AMD's prevalence is complex, as it appears to be equally distributed among all racial groups between the ages of 50–74 but becomes significantly greater in the white population after the age of 75 (Fig. 3.1a). Despite this finding and a linkage between RPE pigmentation and retinal health as suggested by the albinism retinal deficits, the contribution of pigment pathways to AMD remains to be elucidated.

3.2 GPR143 Biology

An important gene involved in the pigmentation pathway encodes a GPCR that is expressed in RPE and melanocytes, GPR143 (Schiaffino 2010). Genetic mutations in the gene encoding GPR143 cause ocular albinism which is associated with the complete retinal albinism phenotype – despite normal or near normal pigmentation in the RPE (Oetting 2002). This observation separates RPE pigmentation from the retinal albinism phenotype, because in individuals with mutations in GPR143, the RPE exhibits normal pigmentation but still develops retinal problems. This directly indicates that the retinal albinism phenotype is linked to GPR143, rather than RPE pigmentation. Further, the ocular albinism phenotype can be rescued by tyrosine hydroxylase activity; tyrosine hydroxylase produces L-dopa but not melanin (Lavado et al. 2006).

We discovered that a ligand for GPR143 is L-dopa, an intermediate product in melanin synthesis produced by the enzyme tyrosinase (Lopez et al. 2008). L-dopa is synthesized by tyrosinase activity and crosses membranes using the same transporter as tyrosine. Thus, L-dopa produced by tyrosinase in pigment granules is released into the RPE cytoplasm and subsequently accumulates in the subretinal space (Roffler-Tarlov et al. 2013). Tyrosinase catalyzes several steps through which tyrosine is hydroxylated creating L-dopa,