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The expression of advanced glycation endproduct receptors in rpe cells associated with basal deposits in human maculas

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Abstract

Basal deposits within Bruch's membrane are associated with aging and age-related macular degeneration (AMD) although the factors causing their formation are incompletely understood. Advanced glycation endproducts (AGEs) accumulate in Bruch's membrane including basal deposits and drusen with aging. One mechanism by which AGEs alter a cell's phenotype is via AGE receptors. The purpose of this study was to immunolocalize and quantify the expression of AGE receptors by RPE cells associated with basal deposits or normal Bruch's membrane that were microdissected from human maculas. Postmortem eyes from 14 aged control donors and five donors with non-neovascular AMD were cryopreserved. RPE cells associated with normal Bruch's membrane or basal deposits were laser capture microdissected. The RNA was extracted and used for RT-qPCR to quantify the expression of RAGE, AGE R1, AGE R2, and AGE R3. Streptavidin alkaline phosphatase immunohistochemistry for these receptors was also performed and sections were bleached from 14 normal and nine AMD donors. RT-qPCR showed significant upregulation of RAGE, AGE R1, and AGE R3 in RPE cells overlying basal deposits compared to cells attached to morphologically normal Bruch's membrane. Immunohistochemical analysis for RAGE, AGER1, R2, and R3 showed diffuse, light staining of RPE cells and strong choriocapillaris staining in areas of normal Bruch's membrane. In areas of basal deposits, the RPE had more intense staining for RAGE and AGER1 compared to regions of normal Bruch's membrane. These results suggest that AGE receptors could influence the formation of basal deposits during aging and AMD.

Keywords

age-related macular degeneration; advanced glycation endproducts; advanced glycation endproduct receptor complex; aging; basal deposits; Bruch's membrane; receptor for advanced glycation endproducts; retinal pigment epithelium

1. Introduction

The accumulation of heterogeneous debris within Bruch's membrane is a histopathologic hallmark of aging and age-related disease to the retinal pigment epithelial-Bruch's membrane-choriocapillaris complex. The composition and location of these deposits

distinguishes chronological aging from age-related disease. Basal laminar deposits, which develop between the RPE cell and basement membrane, are specific for age-related macular degeneration (AMD) when they are thick and contain heterogeneous debris such as long spaced collagen (Sarks, 1976; Newsome et al., 1987; Green and Enger, 1993; van der Schaft et al., 1994; Spraul et al., 1996; Spraul and Grossniklaus, 1997; Curcio and Millican, 1999; Anderson et al., 2001; Johnson et al., 2002; Leu et al., 2002). Basal linear deposits occur within the inner collagenous layer, and are the most specific basal deposit for AMD (Sarks, 1976; Newsome et al., 1987; Green and Enger, 1993; van der Schaft et al., 1994; Spraul et al., 1996; Spraul and Grossniklaus, 1997; Curcio and Millican, 1999; Anderson et al., 2001; Johnson et al., 2002; Leu et al., 2002). Due to their histopathologic importance in defining AMD, determining how these deposits develop may provide new avenues for preventative or early intervention for AMD.

Advanced glycation endproducts (AGEs) are adducts which form through a series of nonenzymatic reactions between sugars and long-lived proteins or lipids. AGEs have been linked to several age-related diseases such as atherosclerosis, Alzheimer's disease, cataracts, and osteoarthritis. Our laboratory previously quantified an age-dependent increase of AGEs in human Bruch's membranes, and immunolocalized AGEs in Bruch's membrane, basal deposits, and drusen (Farboud et al., 1999; Handa et al., 1999). One mechanism by which AGEs induce age-related changes is through interaction with AGE receptors including the receptor for AGEs, or RAGE, and the AGE receptor complex (R1–R3). RAGE can be upregulated with aging and disease, and induces a pathologic response through several signal transduction pathways (Schmidt et al., 2001). The AGE receptor complex forms a complex on the plasma membrane, which is felt in part, to endocytose AGE-modified proteins as a protective response. Downregulation of the AGE R1–3 complex has been associated with disease development (Vlassara et al., 1995; Li et al., 1996). Recently, Howes et al. immunolocalized RAGE and AGEs in the RPE and photoreceptors in early AMD while McFarlane et al. characterized the AGE R complex in bovine and human cultured RPE (Howes et al., 2004; McFarlane et al., 2005). The expression and distribution of these receptors in the RPE-choroid collectively, with respect to aging and disease has not been reported, but might provide insights into the role of AGEs in aging and age-related disease in the fundus. The purpose of this study was to determine the expression pattern and distribution of AGE receptors in the RPE-Bruch's membrane-choriocapillaris complex in human macular samples.

2. Materials and methods

2.1. Tissue processing

The protocol in this study adhered to the tenets of the Declaration for Helsinki regarding research involving human tissue. Globes from 43–95 years old donors were obtained from NDRI (Philadelphia, PA) within 7 hr of death, and were on life support for <24 hr. Eyes of the following donors were used in the study: 14 subjects with a documented history of AMD and 14 aged control donors with no history of chorioretinal disease. The globes were examined with a dissecting microscope. 'Normal' eyes were found to be free of drusen, geographic atrophy, or neovascular disease. 'AMD' eyes were found to have drusen and RPE pigmentary changes, but no geographic atrophy or neovascular AMD. Using RNase free conditions, 6×6 mm calottes of the macula of each subject were obtained for this study. Calottes used for laser microdissection as shown in Table 1A, were cryoprotected using the technique of Barthel and Raymond with slight modification (Barthel and Raymond, 1990). Calottes were progressively infiltrated with sucrose by 30 min incubations at 4 °C in PBS containing 10 and 20% sucrose (w/v). Calottes were then infiltrated in a 2:1 sucrose 20% (w/v):OCT compound (VWR International, Bridgeport, NJ) mixture and frozen at –80 °C. Calottes used for immunohistochemistry were incubated for 1 hr in 2% paraformaldehyde

before sucrose incubation and frozen (Table 1B). All tissue blocks were stored at -80°C until used.

2.2. Tissue sectioning and staining

Macular tissue blocks were sectioned on a cryotome (Leica Microsystems, Inc., Bannockburn, Ill) at $7\text{ }\mu\text{m}$ thickness. For laser capture microdissection, individual sections were fixed in 70, 80, 90, 100% ethanol for 1 min each, and then dehydrated in xylene for 5 min twice. Sections were used immediately for laser capture microdissection. To enhance visualization of Bruch's membrane, some separate sections were stained with 0.5% periodic acid (Sigma) for 5 min and in Schiff's reagent (Sigma) for 10 min.

2.3. Laser capture microdissection

Cells of interest were dissected with an Arcturus PixCell II laser capture microdissector (Arcturus Engineering, Inc., Mountain View, CA) using transfer film (Cap-Sure TF-100; Arcturus Engineering) according to our previously published protocol (Ishibashi et al., 2004). Morphologically normal macular RPE cells were defined using the criteria established by Curcio et al. (1998) and Sarks (1976). Normal macular RPE were defined as having regular cuboidal-columnar cell shape, homogeneous melanin pigmentation, and a height estimated at $10\text{--}15\text{ }\mu\text{m}$ (using the 7.5 and $15\text{ }\mu\text{m}$ spot size of the laser aiming beam). In addition, cells were categorized as normal if they were attached to a non-thickened Bruch's membrane, defined as $<1/4$ RPE cell height and not associated with drusen or other Bruch's membrane abnormality. RPE cells overlying basal deposits had either a regular cuboidal-columnar or a 'flattened' cell shape. Basal deposits were defined as an accumulation of debris in Bruch's membrane such that the thickness was $>1/2$ height of a normal RPE cell. This degree of thickness has been associated with AMD (Sarks, 1976). After dissection, the transfer cap was inspected with the microscope for contaminating tissue, which verified a cleavage plane at the RPE-Bruch's membrane junction, before being placed in $70\text{ }\mu\text{l}$ denaturing buffer that contained 4 M guanidine isothiocyanate, 0.02 M sodium citrate, 0.5% sarcosyl, and $2\text{ }\mu\text{l}$ β -mercaptoethanol (14.5M ; Qiagen Inc, Valencia, CA).

2.4. RNA extraction

Total RNA was extracted from laser captured RPE cells using the RNeasy Micro-kit (Qiagen Inc. Valencia, CA) according to the manufacturer's recommendations. RNA was treated with DNase I (Qiagen, Inc.) during RNA purification. A sample of RPE cells was obtained from a peripheral calotte that was not used for microarray analysis from each donor, which showed preserved 28S and 18S rRNA bands. Before synthesizing probe, RNA quality was assessed by the expression of GAPDH from 100 cells using real time RTPCR with primers designed at the 5' end of the gene.

2.5. Real time RT-qPCR

Total RNA from the equivalent of 200 laser captured RPE cells was reverse transcribed with Sensiscript (Qiagen) in the presence of T4gene32 protein (Ambion Inc., Austin, TX) to enhance first strand cDNA production (Boylan et al., 2001). First strand cDNA was assayed using the LightCycler apparatus (Roche Diagnostics, Nutley, NJ). The primer sequences used in this study were designed using Primer 3 (Whitehead Institute/MIT, Cambridge, MA) and sequences were verified using NCBI Unigene (Table 2). The standard curve consisted of PCR products for the gene of interest using serial dilutions of $5\times 10^{-5}\text{--}5\times 10^{-9}\text{ ng/}\mu\text{l}$. Thermocycling of each reaction was performed in a final volume of $20\text{ }\mu\text{l}$ containing SYBR Green PCR Master Mix ($10\text{ }\mu\text{l}$; Qiagen), Primer A and B ($10\text{ }\mu\text{M}$ each), and $2\text{ }\mu\text{l}$ template DNA in a concentration of 2.5 mM MgCl_2 . The cDNA was denatured at 95°C for 15 min

followed by PCR settings of 94 °C for 15 sec, Tm-5 °C for 20 sec, and 72 °C for x sec where x =PCR product length (bp/20). PCR products were quantified using the second derivative maximum values calculated by the Light-Cycler analysis software. Negative controls without template were produced for each run. Expression levels of all genes were normalized to GAPDH mRNA levels. All PCR products were checked by melting point analysis. For each sample, the experiment was repeated once using different captured cells, and the average expression was used to calculate the expression ratio across samples. The non-parametric Wilcoxon test was used to compare the differential gene expression between RPE cells attached to Bruch's membrane that was unthickened or associated with basal deposits. $P<0.05$ was considered significant.

2.6. Immunohistochemistry and bleaching

Streptavidin alkaline phosphatase (APase) immunohistochemistry was performed on cryopreserved tissue sections using a nitroblue tetrazolium (NBT) development system, as reported previously (Bhutto et al., 2004). In brief, 8 μ m thick cryosections were permeabilized with absolute methanol and blocked with 2% normal goat or rabbit serum in Tris-buffered saline (TBS; pH 7.4 with 1% BSA). Sections were also blocked with an avidin-biotin complex (ABC) blocking kit (Vector Laboratories, Inc., Burlingame, CA). After they were washed in TBS, the sections were incubated overnight at 4 °C with one of the following primary antibodies: mouse anti-human RAGE monoclonal antibody (3 μ g/ml; US Biological, Cleveland, OH; using mouse IgG2a (US Biological) as the isotype control, goat anti-human OST48 polyclonal antibody (2 μ g/ml; Santa Cruz Biotechnology, Inc., Santa Cruz, CA) with goat IgG (Santa Cruz Biotechnology) as isotype control, goat anti-human 80 K-H polyclonal antibody (2 μ g/ml; Santa Cruz Biotechnology) with goat IgG (Santa Cruz Biotechnology) as isotype control, and rabbit anti-human AGE R3 (Galectin-3) polyclonal antibody (5 μ g/ml; Abcam Inc., Cambridge, MA) with rabbit IgG (Vector Laboratories) as isotype control. After they were washed in TBS, sections were incubated for 30 min at room temperature with the appropriate biotinylated secondary antibodies diluted 1:1000 to 1:3000 (Kirkegaard and Perry, Gaithersburg, MD). Sections were incubated with streptavidin APase (1:500; Kirkegaard and Perry), and APase activity was developed with a 5-bromo-4-chloro-3-indoyl phosphate (BCIP)-NBT kit (Vector Laboratories, Inc.), yielding a blue reaction product.

To permit interpretation of immunoreaction product in the RPE and choroid, sections were fixed in 4% paraformaldehyde overnight at 4 °C immediately after streptavidin APase immunohistochemistry. Slides were washed in distilled water at room temperature, immersed in a 0.05% potassium permanganate solution (Aldrich Chemical Co., Milwaukee, WI) for 25 min, and then rinsed in distilled water for 5 min. Sections were covered with 35% peracetic acid (FMC Corp., Philadelphia, PA) in a humidified container for 15–20 min at room temperature followed by washing in distilled water for 10 min.

3. Results

3.1. AGE receptor mRNA expression

Real time RT-qPCR showed that morphologically normal, native macular RPE cells from 14 donors expressed RAGE and AGER1-3. When samples were divided between young (defined as age <60 years old) and old (age >80 years old), no significant expression differences were found. Basal deposits are an established marker for AMD when sufficiently thick, according to Sarks (1976). The expression of RAGE from native RPE cells overlying basal deposits was upregulated 22-fold compared to native RPE attached unthickened Bruch's membrane from normal eyes ($P=0.043$), as shown in Fig. 1. Likewise, AGER1 and AGER3 were up-regulated 15.7-and 6.5-fold, respectively, in RPE cells overlying basal

deposits compared to morphologically normal RPE ($P=0.043$ for AGER1 and AGER3). AGER2 expression did not vary by condition.

3.2. Immunohistochemical localization of AGE receptors

The distribution of AGE receptors in the neurosensory retina has been reported previously, so the analysis in this study focused on the RPE-Bruch's membrane-choriocapillaris (Hammes et al., 1999; Howes et al., 2004). To determine the distribution of RAGE protein in the RPE-Bruch's membrane-choriocapillaris, immunohistochemical evaluation was performed on 23 macular samples. In 'normal' maculas ($n=14$), weak RAGE immunostaining was seen diffusely within nearly all of the RPE cells, but the intensity of staining appeared in a mosaic topographic distribution. Relative to the RPE, strong RAGE immunostaining was seen in the choriocapillaris endothelium and basement membrane (Fig. 2). In AMD maculas containing basal deposits ($n=9$), RAGE stained the RPE diffusely, Bruch's membrane, and the choriocapillaris endothelium and basement membrane. These eyes allowed a comparison between regions with and without basal deposits. In all nine eyes, the relative intensity of the RPE was stronger in RPE cells overlying basal deposits than in areas where Bruch's membrane was not thickened (Fig. 2). The choriocapillaris staining appeared similar between areas with and without basal deposits.

AGE R1 had a similar staining pattern as RAGE. In general, across all ages and in diabetics, AGE R1 localized to the RPE, Bruch's membrane, and choriocapillaris, and displayed stronger staining of the choriocapillaris. In areas of basal deposits, AGER1 localized to the RPE and, relative to the areas without basal deposits within the same sections, displayed stronger immunostaining for AGER1 in the RPE (Fig. 3). AGE R2 showed diffuse staining of the RPE and strong staining of the choriocapillaris in normal samples. In areas of basal deposits in AMD subjects, no differences in staining intensity were observed (data not shown). AGE R3 showed diffuse, light staining of the RPE and stronger labeling of the choriocapillaris across all ages. In areas of basal deposits, no differences in staining intensity were observed in the RPE or choriocapillaris (data not shown).

4. Discussion

Our laboratory previously demonstrated an age-dependent increase of AGEs in human Bruch's membrane, and immunolocalized AGEs to Bruch's membrane including drusen and basal deposits (Farboud et al., 1999; Handa et al., 1999). Our long term hypothesis is that AGEs trigger accelerated RPE aging and promote the transition to age-related macular disease. In mice given an AGE stimulus, we showed ultrastructural aging to Bruch's membrane including basal laminar deposits and transcriptional evidence of aging including induction of inflammation, extracellular matrix expansion, lipid processing abnormalities, cell structure abnormalities, and induction of cell stress molecules by the RPE-choroid (Tian et al., 2005). Beside alterations to the supramolecular structure of Bruch's membrane, AGEs induce an altered cellular phenotype through interaction with a number of receptors. In this study, we evaluated the expression of RAGE and AGER1-3 at the RNA and protein level in human maculas. We found that RAGE, AGER1, and AGER3 mRNAs were up-regulated in RPE cells overlying BDs compared to RPE associated with morphologically normal Bruch's membrane. Our immunohistochemical assessment showed diffuse staining of the RPE, with the suggestion of stronger staining of RAGE and AGER1 in the RPE overlying basal deposits.

Basal deposits are the strongest histopathological marker of aging and age-related disease (Sarks, 1976; Green and Enger, 1993; Spraul et al., 1996; Spraul and Grossniklaus, 1997; Curcio and Millican, 1999). The location and composition of these deposits distinguish aging from AMD. Basal laminar deposits (BlamD), which form between the RPE cell and

basement membrane, are a normal aging change early, but become specific for AMD when they become thick and contain cellular debris, 'long spaced collagen', membranous structures, lipid, and inflammatory proteins (Sarks, 1976; Newsome et al., 1987; Green and Enger, 1993; van der Schaft et al., 1994; Spraul et al., 1996; Spraul and Grossniklaus, 1997; Curcio and Millican, 1999; Anderson and Ozaki, 2001; Johnson et al., 2002; Leu et al., 2002). The most sensitive and specific histopathologic marker of AMD is basal linear deposits (BlinD), which form in the inner collagenous layer of Bruch's membrane. Basal deposits contain a heterogeneous mixture of AGEs, inflammatory proteins, lipid peroxidation products, and cellular debris. Dissecting RPE cells by laser capture microdissection does not allow a determination of gene expression differences between basal laminar and basal linear deposits because this ultrastructural distinction is not resolvable using 8 μ m sections. However, in the experimental design, we chose deposits that were more than 1/2 thickness of an RPE cell, which are associated with age-related macular disease, regardless of whether they are basal laminar or basal linear deposits (Sarks, 1976). In addition, the donors with basal deposits had a known history of AMD.

Clearly, the phenotype of the RPE in vitro is altered by AGE exposure (Handa et al., 1998; Lu et al., 1998; Honda et al., 2001), some of which is likely mediated by AGE specific receptors (Howes et al., 2004; McFarlane et al., 2005). RAGE was upregulated in RPE cells overlying basal deposits. We do not know if AGEs within the basal deposits caused this upregulation. In addition to AGEs, RAGE also binds to other pathogenic molecules such as β -amyloid peptide and S100B/calgranulins. β -amyloid is implicated in the pathogenesis of Alzheimer's disease (Schmidt et al., 2001) and has been localized to drusen (Johnson et al., 2002; Dentchev et al., 2003). The S100/calgranulins are pro-inflammatory polypeptides which have been associated with a number of chronic diseases that have an inflammatory component, such as atherosclerosis, Alzheimer's disease, and diabetes mellitus (Sakaguchi et al., 2003; Kosaki et al., 2004; Lue et al., 2005). Accumulation of RAGE ligands and up-regulation of RAGE results in sustained cellular activation that promotes disease progression (Schmidt et al., 2001). AGEs and S100/calgranulins are potentially important stimulators of RAGE in RPE cells. Howes et al. showed that AGEs and S100B activated cultured RPE cells, and induced apoptosis, a phenomenon seen by the RPE in aging and AMD (Howes et al., 2004). Zhou et al. showed that blue light photo-oxidative stress on A2E in RPE cells induces lipid peroxidation and AGE formation as well as RAGE upregulation (Zhou et al., 2005). These findings suggest that AGEs or other ligands that bind to RAGE are a potential mechanistic link between oxidative stress and induction of inflammation, a potential mechanism of AMD that has gained increased interest due to the recent strong association of polymorphisms in complement factor *H* with AMD (Edwards et al., 2005; Hageman et al., 2005; Haines et al., 2005; Klein et al., 2005; Zarepari et al., 2005).

Howes et al. also showed in human samples, AGE and RAGE immunostaining in the RPE and photoreceptors adjacent to small drusen in early AMD and geographic atrophy, and speculated that RAGE mediated a local inflammatory response that is important in changes associated with AMD (Howes et al., 2004). Our immunohistochemical analysis shows similar findings in an expanded survey, but instead of drusen, we focused on basal deposits. We found stronger staining for RAGE in RPE cells overlying basal deposits than RPE adjacent to normal Bruch's membrane within the same tissue sections. These findings are in agreement with the transcriptional analysis, and are suggestive of a role for RAGE in altering the RPE phenotype in vivo. We observed strong labeling of the choriocapillaris in all samples, but did not find labeling differences in areas containing basal deposits and normal Bruch's membrane. We can't rule out subtle, but important differences in expression of RAGE by the choriocapillaris because immunohistochemistry is an insensitive quantitative assay. Quantitative transcriptional assessment of the choriocapillaris is problematic because it is technically difficult if not impossible to dissect choriocapillaris

endothelium by laser capture microdissection. Our laboratory has demonstrated the feasibility of isolating choroidal endothelial cells from the fundus using immunomagnetic beads, but this technique does not separate choriocapillaris endothelium from endothelium associated with larger choroidal vessels (Wu et al., 2005).

We also found increased mRNA expression of AGE R1 and R3, but not R2, by RPE cells overlying basal deposits compared to cells attached to normal appearing Bruch's membrane. AGER1 (oligosacaryl transferase-48), R2 (80K-H), and R3 (galectin-3) form a functional complex on the plasma membrane (Vlassara et al., 1995; Li et al., 1996). This complex mediates cell specific responses such as synthesis of matrix proteins and cytokines (Pugliese et al., 1997; Seki et al., 2003), and has also been implicated in removing AGE modified proteins by binding, internalizing, and transporting them to the lysosome for degradation. AGE-R1 is thought to bind and endocytose AGE-proteins while AGE-R2 and R3 are involved in AGE ligand binding. Endothelial cells and monocytes upon AGE exposure, upregulate AGE R1 and AGE R3 (Stitt et al., 1999). Similarly, our analysis identified upregulation of AGER1 and R3 mRNA, but no change in R2 mRNA in RPE cells overlying basal deposits. We showed immunohistochemical evidence of increased AGE R1 in RPE cells overlying basal deposits, compared to overlying non-thickened Bruch's membrane, but we did not confirm our transcriptional analysis with immunohistochemical analysis of AGE R3. One possibility is the relative insensitivity of immunohistochemistry compared to RT-qPCR. While our transcriptional analysis for AGE R1 showed a 15.7-fold increased expression by the RPE overlying basal deposits, the differential expression was only 6.5-fold for AGE R3. We presume that this expression difference is insufficient to be recognized with immunohistochemistry. Nevertheless, these findings are also in agreement with McFarlane et al., who found upregulation of AGE-R1 and R3 in RPE cells in vitro after AGE stimulation (McFarlane et al., 2005). The implications of upregulated AGER1 and AGER3 are different from RAGE. AGER3 plays a protective role in renal cells exposed to AGEs while RAGE induces cellular activation (Gugliucci and Bendayan, 1996). Likewise, McFarlane et al. showed that overexpression of AGER3 by RPE cells in vitro reduced VEGF expression while Zhou et al. showed upregulated RAGE and VEGF expression in RPE cells in vitro (McFarlane et al., 2005; Zhou et al., 2005). Due to the defined role of VEGF in neovascular AMD, it is possible that AGER3 is protective while RAGE is mechanistically involved in the development of neovascular AMD. Since AGE modified proteins are degraded by the AGER complex, AGER complex upregulation could be protective against AGE accumulation in Bruch's membrane. Since AGER3 can be secreted, protection could occur by binding soluble AGEs and reduce interaction with AGE receptors (Menon and Hughes, 1999). The upregulation of AGER1 and R3 suggests that the RPE cells overlying basal deposits in this study, were still able to elicit a protective response to AGE exposure.

The relative contribution of phenotypic changes to the RPE by RAGE and the AGER1-3 complex are unknown at this time. This study shows upregulation of both receptors by RPE cells overlying basal deposits, a histopathological marker of age-related disease. The expression of AGE receptors by both the RPE and choriocapillaris may contribute to basal deposit formation. Given the link of these receptors with oxidative stress and inflammation, and the defined roles of these factors in aging and age-related macular disease, further studies appear warranted to clarify the role of these receptors.

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Fig. 1. mRNA expression of AGE Receptors by RT-qPCR. N, normal RPE attached to normal Bruch's membrane; A, RPE overlying basal deposits. The AMD/normal RPE expression ratio with the *P* value is also listed for each receptor.

Fig. 2.

Macular sections from an 85 year old female (donor 9A, Table 1B) incubated with anti-RAGE antibody. A. PAS staining of sections containing morphologically normal RPE and Bruch's membrane (A) and basal deposits within Bruch's membrane (B). Immunostaining with anti-RAGE antibody of unbleached sections containing morphologically normal RPE and Bruch's membrane (C) and basal deposits (D). Immunostaining with anti-RAGE antibody in bleached sections containing morphologically normal RPE and Bruch's membrane (E) and basal deposits (F). The dotted line outlines the bleached RPE. IgG controls of an unbleached section with morphologically normal RPE and Bruch's membrane (G) and a bleached section containing basal deposits (H). The dotted line outlines the bleached RPE. Bruch's membrane (BrM). Choriocapillaris (CC).

Fig. 3. Macular sections from a 93 year old female (donor 12A, Table 1B) incubated with anti-AGE R1 antibody. Immunostaining with anti-AGE R1 antibody of unbleached sections of morphologically normal RPE and Bruch's membrane (A), and basal deposits (B). Immunostaining with anti-AGE R1 antibody of bleached sections containing morphologically normal RPE and Bruch's membrane (C) and basal deposits (D). Dotted line outlines bleached RPE. Bruch's membrane (BrM). Choriocapillaris (CC).

Table 1A

Donors used for laser capture microdissection

Donor	Age (Yrs)	Race	Gender	D-E* (Hrs)	Cause of death
'Normal'					
1N	43	White	M	3:03	Cirrhosis
2N	50	White	M	5:46	Motor vehicle accident
3N	51	White	F	4:42	Lung cancer
4N	52	White	M	5:45	Chondrosarcoma
5N	57	White	F	5:45	Ovarian cancer
6N	59	White	M	6:00	Lung cancer
7N	82	White	M	5:26	Dementia
8N	82	White	M	3:00	Skin melanoma
9N	83	White	F	2:28	Emphysema
10N	83	White	M	2:46	Myasthenia gravis
11N	84	White	M	5:26	Congestive heart failure
12N	84	White	F	4:00	Uterine cancer
13N	87	White	F	3:30	Ovarian cancer
14N	89	White	M	6:00	Myocardial infarction
AMD					
1A	85	White	M	3:25	Respiratory failure
2A	90	White	F	2:15	Stroke
3A	91	White	F	3:30	Pulmonary embolism
4A	91	White	M	2:20	Subdural hematoma
5A	95	White	F	3:15	Alzheimer's disease

* Death to enucleation time.

Table 1B

Donors used for Immunohistochemistry

<i>Donor</i>	<i>Age (Yrs)</i>	<i>Race</i>	<i>Gender</i>	<i>D-E* (Hrs)</i>	<i>Cause of death</i>
'Normal'					
1N	43	W	M	3:03	Cirrhosis
2N	50	W	M	5:46	Myocardial infarction
3N	51	W	F	4:42	Lung cancer
4N	52	W	M	5:45	Chondrosarcoma
5N	57	W	F	5:45	Ovarian cancer
6N	59	W	M	6:00	Lung cancer
7N	82	W	M	5:26	Dementia
8N	82	W	M	3:00	Skin melanoma
9N	83	W	F	2:28	Emphysema
10N	83	W	M	2:46	Myasthenia gravis
11N	84	W	M	6:26	Congestive heart failure
12N	84	W	F	4:00	Uterine cancer
13N	87	W	F	3:30	Ovarian cancer
14N	89	W	M	6:00	Myocardial infarction
AMD					
6A	77	W	M	2:58	Myocardial infarction
7A	82	W	M	3:00	Brain cancer
8A	84	W	F	2:00	Pancreatic cancer
9A	85	W	F	2:28	Multi-organ system failure
10A	87	W	F	3:16	Myocardial infarction
11A	93	W	F	4:00	Multi-organ system failure
12A	93	W	F	5:46	Renal failure
13A	94	W	F	5:35	Cardiac arrest
14A	95	W	F	4:00	Myocardial infarction

* Death to enucleation time

Table 2

Real time RT-qPCR primers

Target gene	Primer name	Sequence	Position	Size (bp)	Annealing Ta (°C)
Glyceraldehyde-3-phosphate dehydrogenase	GAPDH F	CGA CCA CTT TGT CAA GCT CA	987–1006	228	55
	GAPDH R	AGG GGT CTA CAT GGC AAC TG	1195–1214		
Receptor for advanced glycation endproducts	RAGE F	AGG AGC GTG CAG AAC TGA AT	1172–1191	143	55
	RAGE R	GAG TTG GTC TGA GGC CAG AA	1295–1314		
AGE receptor 1	AGER1 F	GTG GGA AAA TGG CAC AAC TT	1520–1539	171	55
(AGER1/DDOST/OST-48)	AGER1 R	CTG GCC ACG TCC CTA TTT TA	1671–1690		
AGE receptor 2	AGER2 F	ACC TCA AGA AGG CAT GAA GC	1738–1757	180	55
(AGER2/PRKCSH/80K-H)	AGER2 R	TAC CCA TCT TTG GAG GCT GT	1898–1917		
AGE receptor 3	AGER3 F	ACC CAC GCT TCA ATG AGA AC	518–537	158	55
(AGER3/galectin3)	AGER3 R	TGC AAC CTT GAA GTG GTC AG	656–675		