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Receptor mediated disruption of retinal pigment epithelium function in acute glycated-albumin exposure

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Abstract

Diabetic macular edema (DME) is a major cause of visual impairment. Although DME is generally believed to be a microvascular disease, dysfunction of the retinal pigment epithelium (RPE) can also contribute to its development. Advanced glycation end-products (AGE) are thought to be one of the key factors involved in the pathogenesis of diabetes in the eye, and we have previously demonstrated a rapid breakdown of RPE function following glycated-albumin (Glyc-alb, a common AGE mimetic) administration in monolayer cultures of fetal human RPE cells. Here we present new evidence that this response is attributed to apically oriented AGE receptors (RAGE). Moreover, time-lapse optical coherence tomography in Dutch-belted rabbits 48 hours post intravitreal Glyc-alb injections demonstrated a significant decrease in RPE-mediated fluid resorption *in vivo*. In both the animal and tissue culture models, the response to Glyc-alb was blocked by the relatively selective RAGE antagonist, FPS-ZM1 and was also inhibited by ZM323881, a relatively selective vascular endothelial growth factor receptor 2 (VEGF-R2) antagonist. Our data establish that the Glyc-alb-induced breakdown of RPE function is mediated via specific RAGE and VEGF-R2 signaling both *in vitro* and *in vivo*. These results are consistent with the notion that the RPE is a key player in the pathogenesis of DME.

Keywords

RPE; DME; diabetes; barrier; AGE; retinopathy; VEGF; rabbit

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1. INTRODUCTION

Diabetic macular edema is the major cause for vision loss in patients with diabetes, affecting more than 75,000 new patients in the United States every year (Ding and Wong, 2012). Although diabetic macular edema is believed to be a microvascular disease originating from the disruption of the inner blood retina barrier formed by retinal endothelial cells (Klaassen et al., 2013), the retinal pigment epithelium is also involved in the development of the disease. The RPE is a single layer of cells separating the choroidal circulation from the neural retina. Two of the quintessential functions of the RPE are: a) the formation of the outer blood retina barrier (BRB); and b) the regulation of normal fluid balance in the outer retina. Nevertheless, due to difficulties in assessing the function of this tissue *in vivo*, the involvement of the RPE in the development and resolution of DME has received only limited attention.

Advanced glycation end-products are thought to be one of the key factors involved in the pathogenesis of diabetes in the eye (Forbes and Cooper, 2013; Tarr et al., 2013). AGEs are a heterogeneous group of molecules formed by non-enzymatic glycation reactions between reducing sugars and free amino groups of proteins, lipids, and nucleic acids (Singh et al., 2001). Although the formation of AGEs is a natural process that occurs with aging and nearly all proteins in the body carry some burden of glycation (Monnier et al., 1992), the rate of AGE formation is accelerated in diabetic conditions due to persistent hyperglycemia and oxidative stress (Baynes and Thorpe, 1999). Several studies in the eye have shown an association between the level of AGE products in the vitreous and the clinical progression of DME (Stitt et al., 1998; Yokoi et al., 2005; Yu et al., 2008). To control appropriate responses, cells have a pattern recognition receptor (RAGE) to detect the levels of AGEs in the environment. Not surprisingly, due to its role in retinal homeostasis, one of the principal targets of AGEs in the eye is the RPE, where RAGE has been shown to be overexpressed in cells adjacent to sub-RPE deposits (Howes et al., 2004). Importantly for the maintenance of outer retina homeostasis, we have recently shown *in vitro* that Glyc-alb (a common AGE mimetic) can disrupt the RPE barrier and increase the permeability of RPE monolayers (Dahrouj et al., 2013). However, the role of Glyc-alb in modulating RPE function *in vivo* has not been tested.

The principal downstream target for AGEs in most tissues, including the RPE, is known to be the secretion of vascular endothelial growth factor (VEGF) (Ma et al., 2007). VEGF is a preeminent endothelial mitogen, inducing the appearance of neovascular blood vessels, which exhibit a tendency to leak, thereby disrupting the inner BRB (Aiello et al., 1994; Kenyon et al., 1996; Ozaki et al., 1997). Similar to AGEs, the level of VEGF is also elevated in the vitreous of patients with DME, and the contributions of this cytokine to the increased leakage of the inner retinal vasculature in diabetes have been well documented (Aiello et al., 1994). We have shown previously that VEGF can also induce a breakdown of the RPE barrier *in vitro* (Ablonczy and Crosson, 2007; Ablonczy et al., 2011; Ablonczy et al., 2009) supporting the idea that in addition to affecting the retinal vasculature, VEGF can also modulate RPE function.

To assess RPE fluid resorption and barrier function *in vivo*, we have recently developed a new methodology and have shown that VEGF induces a dramatic reduction in the rate of normal subretinal fluid resorption (Dahrouj et al., 2014). Although these optical coherence tomography (OCT) studies provided evidence of a potentially crucial role for VEGF in regulating RPE function *in vivo*, the experiments required the subretinal administration of higher than physiological amounts of VEGF. Therefore, we considered a more relevant model of the diabetic eye; one based on a high level of Glyc-alb in the vitreous, to assess changes in the function of the RPE *in vivo*. Using this animal model as well as fetal human RPE (fhRPE) cell cultures, the current studies provide new evidence that the Glyc-alb-induced breakdown of RPE function is mediated by RAGE and VEGF-R2. These results are in agreement with the idea that the RPE is a key player in the pathogenesis of DME.

2. METHODS

2.1. Tissue culture

ARPE19 cells were obtained from the American Type Culture Collection (Manassas, VA) and hRPE cells were isolated from human fetal eyes acquired from Advanced Bioscience Resources (Alameda, CA). hRPE cells from three different donors were used throughout the experiments. Confluent monolayers of both cell types were established and maintained on permeable membrane inserts (Costar Clear Transwell, 0.4 μm pore, 24 mm; Thermo Fisher Scientific, Fair Lawn, NJ) as described previously (Ablonczy et al., 2011). An STX2 electrode-equipped epithelial volt/ohm meter (World Precision Instruments, Sarasota, FL) was used to assess transepithelial electrical resistance (TEER). Resistance values for each condition were determined from a minimum of four individual cultures and corrected for the inherent Transwell resistance within 3 minutes after removing the plates from the incubator. Only confluent monolayer cultures with stable TEER values were used in these experiments (40-50 $\Omega\cdot\text{cm}^2$ for ARPE 19 cells and $> 600 \Omega\cdot\text{cm}^2$ for hRPE cells). All values represent mean $\pm$ SE. Data were analyzed using ANOVA, and were considered statistically significant at $p < 0.05$.

2.2. Cell treatments

Cultures were treated apically with 100 $\mu\text{g/mL}$ of human albumin (Alb) or human Glyc-alb (both from Sigma-Aldrich, St. Louis, MO) diluted in phosphate-buffered saline (PBS). Change in TEER was followed at the time of administration and for six hours post administration. In selected experiments, the RAGE antagonist, FPS-ZM1 (100 nM; Millipore, Billerica MA) or the selective VEGFR2 inhibitor, ZM323881 (10 nM; Tocris Bioscience, Ellisville, MO) was administered apically to the cells one hour prior to Alb or Glyc-alb.

2.3. Immuno-histochemistry

Monolayers of hRPE cells were analyzed with immunohistochemistry following methods described previously (Ablonczy et al., 2011; Ablonczy et al., 2009; Dahrouj et al., 2013). The primary antibodies were mouse anti-ZO-1 (diluted 1:100; Chemicon, Temecula, CA) and rabbit anti-RAGE (1:100; Sigma). The secondary antibodies were Alexa 594-conjugated goat anti-rabbit (diluted 1:500; Fisher) and Alexa 488-conjugated goat anti-mouse (1:500;

Fisher). Slowfade (antifade with DAPI; Fisher) was used to visualize cell nuclei. Samples were analyzed in an FV10i-LIV inverted confocal microscope (Olympus, Tokyo, Japan). Stacks of 25 confocal images were collected at successive focal planes (1 μm apart) throughout the entire cell monolayer (24 μm).

2.4. Immunoblots

Immunoblots were performed on human (cadaveric eye obtained from an eye bank, San Diego, CA), and rabbit tissues, as well as on cultures of RPE cells. To prepare the ocular tissues, the anterior part of the eye and the retina were removed and the RPE/choroid was gently peeled away from the sclera. These tissues were washed in ice-cold PBS, and then lysed in buffer (pH 7.5; 2.42 g/L Tris Base, 1 mM EGTA, 2.5 mM EDTA, 5 mM dithiothreitol, 0.3 M sucrose, 1 mM Na_3VO_4 , and 20 mM NaF – all from Sigma; one complete mini protease-8 inhibitor tablet; Roche Applied Science, Indianapolis, IN). To prepare cell culture lysates, monolayers of cells were washed with ice-cold PBS and lysed in the above lysis buffer, scraped, and collected in a centrifuge tube. All samples were sonicated twice for 10 seconds each, centrifuged for 5 min at 10,000g, and the supernatant was collected. Equal quantities of the samples (determined by protein assay; Bio-Rad, Hercules, CA) were separated on 4-12% Bis-Tris gel, transferred to a blotting membrane, blocked with 5% nonfat dry milk, and incubated with monoclonal mouse anti-RAGE or mouse anti- β actin (Sigma) overnight at 4°C. After washing, HRP-conjugated secondary antibody was administered for two hours and the lanes visualized with a VersaDoc 5000 imager (Bio-Rad) after treatment with chemiluminescent reagent (Fisher).

2.5. Animals

Twenty-four Dutch-belted rabbits weighing 1.5-2 kg were anesthetized using intramuscular ketamine (20 mg/kg) and xylazine (2 mg/kg) (both from Butler Schein, Columbus, OH), with additional aliquots administered as needed during extended experiments. The pupils were dilated using 10% phenylephrine hydrochloride (Bausch & Lomb, Tampa, FL) and atropine (Bausch & Lomb) drops administered topically. For local anesthesia, proparacaine (0.5%; Bausch & Lomb) drops were administered topically before and during the procedure. Animal handling was performed in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research; and the study protocol was approved by the Animal Care and Use Committee at the Medical University of South Carolina.

2.6. Intravitreal and subretinal bleb injections

For intravitreal administration, a 30-gauge needle was inserted 2-3 mm posterior to the limbus and 10 μL of Alb or Glyc-alb was injected (both at a concentration of 1 mg/mL). In selected experiments, FPS-ZM1 (1 μM) or ZM323881 (1 μM) was co-administered with Alb or Glyc-alb.

Forty-eight hours after the intravitreal administration, one subretinal bleb per eye was generated using an injection of PBS following methods described previously (Dahrouj et al., 2014). In short, after setting the intraocular pressure at 10 mmHg via anterior segment cannulation, a 26-gauge needle was inserted 2-3 mm posterior to the limbus and a 33-gauge blunt needle was advanced through the larger needle across the vitreous under the guidance

of a Spectralis OCT instrument (Heidelberg Engineering, Heidelberg, Germany). Reaching the sub-retinal space, 1-5 μ L of sterilized PBS was gently injected to produce a bleb. Infrared fundus images and OCT tomograms (both in transverse and sagittal directions) as well as full volume scans were taken every ten minutes over the course of one hour using the Spectralis OCT instrument. Rates of bleb resorption were calculated using Graphpad Prism 6 software by linear regression analysis of the changes in bleb volume, as previously described (Dahrouj et al., 2014). All values represent mean $\pm$ SE. Data were analyzed using ANOVA, and were considered statistically significant at $p < 0.05$.

3. RESULTS

3.1. RAGE mediates Glyc-Alb responses in fhRPE cells

We had previously shown that Glyc-alb induces a reduction in TEER *in vitro* (Dahrouj et al., 2013); therefore, we were interested in identifying the corresponding receptor. First, the expression of RAGE (a principle receptor for AGEs) was assessed using Western blot analysis in cell cultures as well as human and rabbit eye tissue. Mouse monoclonal anti-RAGE showed the presence of a band at the expected molecular weight (~50 kDa) in RPE/choroid lysates from rabbit and human tissue as well as in both ARPE-19 and fhRPE cell cultures (**Fig. 1A**). Although the band was expressed in both tissues and cells, the expression of the protein was elevated in the native tissue (and even more so in the human) compared to the cell cultures. In some samples, a second band (55 kDa) was also present, known to be an isoform of the receptor (Neeper et al., 1992).

To assess the functional involvement of RAGE in mediating the pharmacological response, we utilized a recently developed RAGE antagonist, FPS-ZM1 (**Fig. 1B**). As basal Glyc-alb has not elicited responses in our previous experiments (Dahrouj et al., 2013), all chemicals were applied apically. The administration of 100 μ g/mL of Glyc-alb caused a $25 \pm 3\%$ decrease in TEER after six hours, which was significantly lower when compared to treatment of control cultures with the same concentration of Alb. One hour pretreatment with FPS-ZM1 (100 nM) before the administration of Glyc-alb, however, prevented any TEER decrease measured after six hours. The administration of 100 nM FPS-ZM1 one hour prior to Alb treatment did not significantly affect the TEER.

3.2. The localization of RAGE

Our previous experiments showed that Glyc-alb induced a breakdown of the RPE barrier only when administered apically (Dahrouj et al., 2013). The localization of RAGE in the RPE was investigated using immunohistochemistry and confocal microscopy in fhRPE cell monolayers, which mimic most properties of the native RPE (Ablonczy et al., 2011) but are free from interference of other ocular tissues. **Figure 2A** shows that the transverse and sagittal Z-stack profiles localized RAGE (red) close to ZO1 (green) which is at the apical/basolateral junction and well above the basolaterally localized nucleus (blue). Analysis of the consecutive images in the stack for mean color pixel intensities (**Fig. 2B**) provides additional evidence that the peak for RAGE staining was 3 μ m from the apical surface, while the peaks for ZO-1 and cell nuclei were 4 μ m and 9 μ m below the apical surface, respectively.

3.3. VEGF-R2 inhibition prevents the Glyc-alb-response on the RPE barrier

AGE administration has previously been reported to induce VEGF secretion from RPE cells (Ma et al., 2007). To determine whether VEGF-R2 activation is responsible for the action of Glyc-alb on the RPE barrier in our experimental system, we used a relatively specific inhibitor, ZM323881. Cells were treated with ZM323881 one hour prior to the administration of Glyc-alb and measured the resulting TEER of the monolayers six hours post Glyc-alb administration.

When ARPE19 cells were administered 100 µg/mL Glyc-alb, a significant ($25 \pm 3\%$) decrease in TEER was measured compared to treatment with the same concentration of Alb (**Fig. 3A**). One hour pretreatment with 10 nM of ZM323881 blocked the effect of Glyc-alb on TEER. However, in control cultures receiving 100 µg/mL of Alb pretreatment, ZM323881 had no significant effect on TEER. Similar results were measured with primary culture of hRPE cells (**Fig. 3B**), where Glyc-alb administration resulted in a significant $23 \pm 3\%$ decrease compared to treatment with the same concentration of Alb. Again, pretreatment with 10 nM ZM323881 reversed the Glyc-alb-induced reduction in TEER but no significant effect was observed on TEER after co-administering ZM323881 with Alb.

3.4. Administration of Glyc-alb reduces fluid resorption by the RPE

To assess the effects of RAGE agonists on the function of the RPE *in vivo*, we injected 1 mg Glyc-alb or normal Alb at a final concentration of approximately 1 mg/mL into the vitreous humor of rabbits. Intravitreal Glyc-alb was expected to diffuse through the neural retina to activate the RAGE receptors on the apical surface of the RPE (**Fig. 2**). Forty-eight hours after the administration of Alb, a PBS-filled subretinal bleb was created and monitored using OCT scans over the course of one hour to determine the rate of bleb resorption. As shown in **Figure 4A**, bleb resorption in the Alb-injected eye was linear and the volume decreased by $28 \pm 2\%$ in one hour. In Glyc-alb-injected eyes, no significant bleb resorption was measured during the experimental period. In control animals that received intravitreal Alb, subretinal blebs resorbed at a rate of $6.2 \pm 1.2 \mu\text{L}/\text{cm}^2\text{h}$. In animals injected with Glyc-alb, a significantly slower resorption rate ($2.2 \pm 0.4 \mu\text{L}/\text{cm}^2\text{h}$) was measured when compared to control Alb-injected animals (**Fig 4B**).

To confirm that similar to the reduction of TEER *in vitro* (**Fig 1B**) the RPE response to intravitreal Glyc-alb was dependent on the RAGE receptor, we chose to perform the analogous experiment with the RAGE antagonist in the rabbit model. FPS-ZM1 (1 µM, 328 ng compound total in the vitreous) was co-administered with either Alb or Glyc-alb. Two days later, PBS was injected subretinally and RPE fluid resorption rates were measured. Eyes co-administered with Glyc-alb and FPS-ZM1 showed bleb fluid resorption rates of $8.6 \pm 0.3 \mu\text{L}/\text{cm}^2\text{h}$, effectively reversing the Glyc-alb response (**Fig 4B**). This rate was not significantly different from the rates in control eyes receiving Alb alone (see above) or Alb and FPS-ZM1 together ($7.0 \pm 1.1 \mu\text{L}/\text{cm}^2\text{h}$), indicating a RAGE-specific response to Glyc-alb.

To determine if the *in vivo* response to Glyc-alb also involves activation of VEGF-R2, ZM323881 was again employed (**Fig. 4B**). Co-injection of 1 µM of ZM323881 (411 ng

compound total in the vitreous) with Glyc-alb intravitreally resulted in a restoration of normal RPE function with a fluid resorption rate of $6.3 \pm 0.9 \mu\text{L}/\text{cm}^2\text{h}$ that was not significantly different from rates measured in Alb-injected control eyes. The co-injection of ZM323881 with Alb did not significantly affect the rate of fluid resorption ($8.2 \pm 1.5 \mu\text{L}/\text{cm}^2\text{h}$) compared to eyes injected with either Alb injection alone or the co-injection of ZM323881 and Glyc-alb.

4.0. DISCUSSION

The loss of visual acuity in diabetic retinopathy is most often secondary to the development of DME (Bandello et al., 2003; Frank, 2004). The clinical progression of diabetic retinopathy as well as diabetic macular edema correlates with the concentration of VEGF and AGE-products in the vitreous (Yokoi et al., 2005), both of which are primarily thought to disrupt the inner BRB leading to accumulation of fluid within the retina (Antonetti et al., 1999; Stitt et al., 2000).

The breakdown of the inner BRB is known to be important for the development of edema, but several lines of evidence show that the RPE is also involved. The RPE forms the outer BRB, which is responsible for the resorption of extracellular fluid from the retina to the choroid, and the RPE is also essential for keeping the retina in a relatively dehydrated state (Marmor and Negi, 1986). However, the role of RPE barrier in the pathogenesis of DME has received limited attention before, primarily due to the difficulty of assessing RPE function under pathologic conditions in lack of a proper animal model. Nevertheless, a leakage of FITC-dextran has been demonstrated through the RPE barrier in diabetic rodents (Xu and Le, 2011). More recently, our analysis of subretinally injected blebs by OCT in the merangiotic rabbit retina allowed a direct assessment of RPE function independent of the vasculature (Dahrouj et al., 2014). Taken together, these studies have shown that the RPE has a potentially key role in the development of ocular symptoms of diabetes.

Although our technique provided convincing evidence regarding the pathophysiological role of VEGF in RPE barrier breakdown, high concentrations of VEGF in subretinal blebs are not an appropriate model for diabetic changes in the retina. Therefore, we set out to evaluate an improved model of diabetic eye disease, based on the exogenous administration of intravitreal RAGE agonist to mimic the endogenous accumulation of AGE in the diabetic eye. We hypothesized that even in otherwise non-pathological eyes, elevated levels of AGE products might lead to an increased release of endogenous VEGF, which in turn could modulate RPE function. In this model we utilized Glyc-alb as an AGE receptor ligand at concentrations previously reported in the literature (Takehashi et al., 2008) and non-glycated albumin to control for any changes induced by increase oncotic pressure associated with the intravitreal injections.

The studies presented in this manuscript establish that intravitreal Glyc-alb disrupts the function of the RPE *in vivo* and decrease the rate of subretinal fluid resorption. The RPE expresses several pattern-recognizing receptors activated by AGEs, such as RAGE, AGE-R1, AGE-R2, AGE-R3, (McFarlane et al., 2005) as well as CD36 (Ryeom et al., 1996; Thornalley, 1998) and scavenger receptor type I and II (Duncan et al., 2002; Thornalley,

1998). Immunoblots confirmed that RAGE is indeed expressed in the human and rabbit RPE, as well as in ARPE19 and fhRPE cells; and confocal microscopy provided evidence that in agreement with our TEER measurements (Dahrouj et al., 2013), RAGE is localized apically in the RPE. Previous immunohistochemical studies indicated a perinuclear arrangement for other AGE receptors (McFarlane et al., 2005). *In vivo* studies, showing RAGE to correlate with AGE in the basal deposits under the RPE (Yamada et al., 2006), implied an RPE response to deposit formation. The apical localization found in the current study is, however, consistent with the ability of intravitreal Glyc-alb to decrease the rate of resorption in the rabbits and also with clinical observations that diabetic retinopathy is associated with elevated levels of AGE in the vitreous (Yamagishi et al., 2006; Yokoi et al., 2005). An increase in intravitreal AGE is plausible either as leakage through inner retina vessels or through AGE that can be generated in the retina *in situ*. In this scheme, the disruption of the RPE barrier allows a positive feedback, causing further increase in AGE accumulation and more damage in the ocular environment until macroscopic pathology is observed.

AGEs are known to induce VEGF secretion and expression both in the RPE (Ma et al., 2007) and endothelia (Pedram et al., 2002); moreover, the action of VEGF on the RPE barrier is mediated by VEGF-R2 both *in vitro* (Ablonczy and Crosson, 2007) and *in vivo* (Dahrouj et al., 2014). Therefore, we tested here if a relatively selective VEGF-R2 inhibitor could reverse the AGE effect on the RPE. We found that the inhibitor blocked the effect of Glyc-alb on RPE function both *in vitro* and *in vivo*, confirming the importance of the VEGF/VEGF-R2 pathway in AGE responses of the RPE. Representing a potential limitation to our studies, commercially available Glyc-alb was shown to contain high levels of amadori-glycated (non-AGE) products, which could result in off-target effects (Higai et al., 2006). However, in our results the Glyc-alb-induced dysfunction was completely reversed using the specific novel RAGE antagonist FPS ZM1 (Deane et al., 2012), providing evidence that the Glyc-alb-response was RAGE receptor specific and not a result of off-target pathways.

The molecular mechanisms behind DME are not clearly understood and there are only limited traditional treatment options available (such as steroids and laser photocoagulation) with some serious possible side-effects (Bakri and Kaiser, 2005; Bandello et al., 2001; Kiernan and Mieler, 2009). The leading treatment today is based on monoclonal antibody fragments against VEGF (Bandello et al., 2012; Ho et al., 2012) or its receptors (Stewart et al., 2012). Besides being expensive, anti-VEGF treatment is also prone to side-effects as VEGF is both neuroprotective (Kurihara et al., 2012; Nishijima et al., 2007; Saint-Geniez et al., 2008) and required for choroidal health (Gibran et al., 2007; Oishi et al., 2013). Preclinical studies targeting upstream events, such as the suppression of AGE formation or RAGE-signaling showed encouraging initial results; however, subsequent clinical trials appeared to exhibit disappointing side effects (Peyroux and Sternberg, 2006). The development of this model of DME will make it possible to better understand the mechanisms of DME, and in the future assist with developing new treatments.

The experiments presented in this manuscript represent a very powerful method to measure RPE function *in vivo*. Nevertheless, one limitation is that they are based entirely on assessing subretinal fluid resorption without a direct visualization of RPE barrier disruption.

Linking the resorption of fluid to RPE barrier breakdown is an important element of our conceptual framework. Although we did not evaluate RPE barrier disruption in the current manuscript, we have shown that the VEGF-induced reductions in the rate of fluid resorption is best explained by a RPE breakdown (Dahrouj et al., 2014). As the experiments presented in this study link Glyc-alb-induced reductions in fluid reabsorption to VEGFR2 receptor activation, the reduction in fluid reabsorption likely involves RPE barrier disruption. However, other mechanisms cannot be excluded. Understanding the precise cellular mechanisms and signaling pathways behind these our observations will require additional investigations.

In summary, our studies provided new evidence that intravitreal AGEs can acutely modulate RPE function in the eye, creating conditions that favor the accumulation of extracellular fluid. These actions of AGE appear to be mediated through the RAGE receptor and involve the activation of the VEGF/VEGF-R2 pathway in the RPE. Although our studies utilized simulated conditions of a diabetic eye, diabetes is not the only disease where subretinal fluid can endanger vision. Subretinal fluid is a common symptom in various conditions, such as age-related macular degeneration, venous occlusive disease, uveitis, eye surgeries, injuries, and certain forms of retinitis pigmentosa (Tranos et al., 2004). Many of these diseases do not exhibit vascular phenotypes characteristic of diabetes, nevertheless, fluid can still accumulate. Thus, further study of the retina may be a key to not only developing new and more promising treatments for DME but also to better understanding of overall ocular health.

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Abbreviations

Alb	albumin
AGE	advanced glycation end-products
BRB	blood-retina barrier
DME	diabetic macular edema
Glyc-alb	glycated-albumin
fhRPE	fetal human RPE
OCT	optical coherence tomography
PBS	phosphate-buffered saline
RAGE	AGE receptor
RPE	retinal pigment epithelium

TEER	transepithelial resistance
VEGF	vascular endothelial growth factor
VEGF-R2	VEGF receptor type 2

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- A model of diabetic eye disease using glycated-albumin (Glyc-alb) in DB rabbits and fhRPE cells.
- Assessment of RPE function *in vivo* using kinetics of subretinal fluid resorption.
- Glyc-alb decreases subretinal fluid resorption 48 hrs post intravitreal injection.
- Glyc-alb response is mediated by RAGE and VEGF-R2 *in vivo* and *in vitro*.
- The RAGE receptors are localized on the apical surface of the RPE cells.

Figure 1. RAGE inhibition prevents the Glyc-alb-induced reduction in TEER

A, Western blot analysis for RAGE showing a band at 55 kDa (the expected molecular weight) in ARPE19 and hRPE cell culture homogenates, as well as in isolated rabbit and human RPE/choroid tissue. **B**, Administration of 100 μ g/mL Glyc-alb to ARPE-19 cells caused a significant decrease in TEER at six hours post treatment compared to the administration of the same concentration of Alb. One hour pretreatment with 100 nM FPS-ZM1 (a relatively specific RAGE antagonist) reversed the effect of Glyc-alb; however, FPS-ZM1 had no significant effect on Alb. Values represent means $\pm$ SE of individual measurements normalized to the average TEER values at $t = 0$ min analyzed by ANOVA. * $p < 0.05$; $n = 6$

Figure 2. The localization of RAGE in the RPE

A, Average projection of a confocal Z stack as well as transverse and sagittal cross-sections from fhRPE cell monolayers. Red immunohistochemical staining (Alexa 594) represents RAGE, green (fluorescein) is for ZO-1, and blue (DAPI) shows the cell nuclei. The apical (a) and basolateral (b) surface of the cells is indicated using small white arrows in the cross-sections. bar = 20 μm **B**, The analysis of mean pixel intensities for the consecutive images in the stack indicates that RAGE staining is apical compared to both ZO-1 and the cell nuclei.

Figure 3. VEGF-R2 inhibitors reverse AGE action on RPE function

Treatment of monolayer cultures of (A) ARPE19 and (B) fhRPE cells with Glyc-alb resulted in significant decrease in TEER at six hours post administration. Pretreatment with 10 nM ZM323881 one hour prior to treatment with Glyc-alb reversed its effects on the TEER. ZM323881 showed no significant effect on TEER when Alb was co-administered. Values represent means $\pm$ SE of individual measurements normalized to the average TEER at $t = 0$ min analyzed by ANOVA. * $p < 0.05$; $n = 6$.

Figure 4. The effect of AGEs on RPE fluid resorption in vivo

A, The change in subretinal saline blebs at volume flux over the course of one hour. Before the subretinal injections, the eyes were incubated for 2 days with 1 mg/mL albumin (Alb) or glycated albumin (Glyc-alb). Blebs in eyes incubated with Alb exhibited a significant reduction in volume by 30 min after bleb creation; however, no such reduction was observed over the course of one hour in eyes treated with Glyc-alb. Values represent means $\pm$ SE of individual measurements normalized to initial values at $t = 10$ min analyzed by Student's t -test. *** $p < 0.001$; $n = 4$. **B**, Intravitreal incubation with 1 mg/mL Glyc-alb for two days resulted in a significant decrease in the rate of RPE fluid resorption compared to incubation with normal Alb. Intravitreal co-administration of 1 mg Alb or Glyc-alb and the FPS-ZM1 RAGE antagonist (1 μ M) prevented the decrease in resorption rate seen when Glyc-alb was administered alone. Co-administration of 1 μ M ZM323881 with Glyc-alb also reversed the ability of Glyc-alb to decrease RPE fluid resorption. FPS-ZM1 nor ZM323881 had no significant effect on RPE fluid resorption when co-administered with Alb compared to single Alb treatments. Values represent means $\pm$ SE of individual measurements analyzed by ANOVA. $n = 3 - 5$; * $p < 0.05$