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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 h 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.

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

Abbreviations: Alb, albumin; AGE, advanced glycation end-products; blood–retina barrier, BRB; 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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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, 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 hfRPE cells were isolated from human fetal eyes acquired from Advanced Bioscience Resources (Alameda, CA). hfRPE 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 min after removing the plates from the incubator. Only confluent monolayer cultures with stable TEER values were used in these experiments (40–50 Ω cm^2 for ARPE19 cells and >600 Ω cm^2 for hfRPE cells). All values represent mean ± 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 μg/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 hfRPE cells were analyzed with immonohistochemistry following methods described previously (Ablonczy et al., 2011, 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 s each, centrifuged for 5 min at 10,000*g*, 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.