Matrix Metalloproteinase Activity Creates Pro-Angiogenic Environment in Primary Human Retinal Pigment Epithelial Cells Exposed to Complement

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PURPOSE. Mechanistic studies have shown that inflammation, complement activation, extracellular matrix (ECM) turnover, growth factor imbalance, and oxidative stress are fundamental components of age-related macular degeneration (AMD). Matrix metalloproteinases (MMPs) mediate ECM turnover but also process various bioactive molecules. Here, we tested whether complement attack on RPE monolayers changes MMP secretion and activation, thereby altering the availability of growth factors in the extracellular space.

METHODS. Human embryonic RPE monolayers with stable trans-epithelial resistance (TER) were established. Complement activation was induced with H₂O₂ and normal human serum. MMP-2/9, vascular endothelial growth factor (VEGF) and pigment epithelium-derived factor (PEDF) protein, and mRNA levels were analyzed by Western blotting, ELISA, and real-time PCR; activity of MMP-2/9 by gelatin zymography.

RESULTS. Complement activation resulted in a loss of TER, which required transient membrane attack complex formation, activation of the alternative pathway, and VEGF secretion and signaling. Despite the generation of reactive oxygen species, cellular integrity or intracellular adenosine triphosphate (ATP) levels were unaffected. However, expression of MMP-2/9 and their protease activity was elevated. Inhibition of MMP-2/9 activity increased PEDF and decreased VEGF levels in the apical and basal supernatants but had no effect on their expression levels. VEGF levels in the supernatant correlated with the level TER reduction.

CONCLUSIONS. These studies suggest that complement activation, by altering the expression and activation of MMPs, has the ability to generate a proangiogenic environment by altering the balance between VEGF and PEDF. Our findings link reported results that have been associated with AMD pathogenesis; oxidative stress; complement activation; VEGF/PEDF ratio; and MMP activity. (*Invest Ophthalmol Vis Sci.* 2012;53:1953–1961) DOI:10.1167/iovs.11-8638

Age-related macular degeneration (AMD) is characterized by a progressive loss of central vision resulting from damage to the photoreceptor cells in the central area of the retina, the macula. AMD occurs in two forms: wet and dry; with the dry form making up 80% to 90% of total cases.^{1,2} Dry AMD involves atrophy to the retinal pigment epithelium (RPE) followed by the slow degeneration and atrophy of the photoreceptors in the macula by mechanisms not fully understood. Wet AMD on the other hand is associated with choroidal neovascularization (CNV) in the center of the retina. Since new vessels are leaky, the resulting fluid accumulation can cause retinal detachment, concomitant with rapid photoreceptor loss. What both forms have in common, however, is pathology at the RPE/choroid interface, which includes a thickening of Bruch's membrane (BrM), due to the deposition of extracellular material between the RPE and BrM (sub-RPE deposits and drusen).

The RPE is a monolayer of hexagonally arranged, highly pigmented cells, located between the neural retina and the choroid, and forming part of the blood-retina barrier. Its many functions include (reviewed in Ref. 3): the absorption of light that did not get captured by the photoreceptor outer segment pigments; epithelial transport of molecules (nutrients, ions, water, and metabolites) between the subretinal space and the choroidal blood supply; spatial ion buffering; re-isomerization of the chromophore 11-*cis*-retinal from all-*trans* retinal; the daily removal of photoreceptor outer segments by phagocytosis; the secretion of molecules such as growth factors, proteases, and others that control the stability of the photoreceptor cells, BrM and the choroid; and finally, the modulation of the immune response, since the RPE participates in control of the immune privilege in the healthy eye or the mounting of an immune response in the diseased eye. Abnormalities in any of these processes might participate in RPE cell pathology.

The complement system is an essential part of the innate immune system. While its main role is to eliminate foreign antigens and pathogens as part of the normal host response (reviewed in Refs. 4 and 5), inappropriate or excessive complement activation has recently been shown to be involved in the pathogenesis of a number of different autoimmune, inflammatory, and ischemic disease states (reviewed in Ref. 6). The complement system can be activated through three different pathways: the classical (CP), lectin (LP), and alternative pathway (AP).⁷ Its activation components serve different purposes. C3b opsonizes molecules and cell surfaces to target them for removal by phagocytes. The anaphylatoxins C3a and C5a promote inflammation by attracting macrophages, neutrophils, natural killer and B- and T-lymphocytes. Lastly, the membrane attack complex (MAC) can trigger cell lysis or in the case of limited complement activation, a sublytic attack. At sublytic doses, the complement MAC complex has a wide range of effects on many cell types leading to changes in cellular responses such as secretion, adherence, aggregation,

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Supported in part by the National Institutes of Health Grant R01EY019320; Department of Veterans Affairs Grant I01 RX000444; Foundation Fighting Blindness; and an unrestricted grant to MUSC from Research to Prevent Blindness, New York, New York (RPB). BR is a Research to Prevent Blindness Olga Keith Wiess Scholar. Animal studies were conducted in a facility constructed with support from the National Institutes of Health Grant C06 RR015455.

Submitted for publication September 21, 2011; revised December 13, 2011, and February 7, 2012; accepted February 9, 2012.

Disclosure: M. Bandyopadhyay, None; B. Rohrer, Taligen Therapeutics (C), P, Alexion Pharmaceutical which bought out Taligen Therapeutics (R)

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chemotaxis, cell division, or membrane function (reviewed in Ref. 8; examples in Refs. 9–12). Complement-activation has been hypothesized to be involved in the inflammatory response leading to AMD pathology (e.g., Refs. 13–15). This hypothesis was based on the observations that complement components have been found to be associated with the pathological features of AMD. Drusen contain, among other proteins, complement components; and both BrM and the RPE were found to be immunopositive for complement regulatory proteins, cell-bound complement component C3 activation fragments, as well as MAC proteins.^{13,16–18} Interestingly, MAC deposition has been shown to be the highest in the macula, and MAC staining intensity appears to be correlated with AMD severity and the loss of RPE cells.¹⁶ The complement-mediated inflammation hypothesis has been strengthened by the genetic linkage data, which shows that polymorphisms in the AP control protein factor H,^{14,19–21} and other complement genes (*CFB*, *C2*, and *C3*^{22,23}), are strongly associated with all forms of AMD. Finally, recent evidence provided in both the CNV mouse model of wet AMD^{24,25} and the RPE monolayers^{26,27} demonstrated that complement activation is involved in controlling VEGF mRNA expression, protein levels, as well as secretion.

Matrix metalloproteinases (MMPs) are a family of at least 20 zinc endopeptidases that take part in the regulation of cell matrix composition by cleaving basal lamina and extracellular matrix (ECM) proteins. MMPs can be secretory or cell surface bound. Under normal conditions, MMP activity is required for tissue remodeling, but altered MMP activity has been reported in disease. Most MMPs are secreted as inactive pro-proteins but get activated when cleaved by extracellular proteinases. The RPE is known to produce MMPs, including the gelatinase enzymes MMP-2 and MMP-9,²⁸ MMP-1 (interstitial collagenase) and MMP-3 (stromelysin-1),²⁹ as well as MMP-14 (type I transmembrane MMP).³⁰ One of the targets of MMP activity is the ECM molecules in BrM.³¹ BrM is a complex pentalaminal structure composed of the RPE basement membrane on its apical side, and the choriocapillaris basement membrane on its basal side. The center consists of two collagenous zones containing collagen types I, III, IV, and V, divided by an elastic layer. Alterations in the solubility of the collagens occur with age.³² Since MMP-2 and MMP-9 preferentially degrade basement membrane components such as type IV collagen, and the levels of MMP-2 and MMP-9 increase with aging in both the macula and the periphery,³³ special attention has been paid to those two MMPs in AMD (e.g., Ref. 34). A second set of targets for MMP activity are growth factors; matrix-bound VEGF appears to get mobilized by MMP, in particular MMP-9,³⁵ whereas PEDF is a substrate for MMP-2 and MMP-9.³⁶ It is unclear what controls MMP secretion from the RPE and subsequent activation; although angiogenic factors produced in CNV membranes have been shown to increase secretion of MMP-2 and MMP-9 by RPE cells, with activity documented by Hoffman et al.,²⁸ while oxidative stress has been shown to increase MMP-1 and MMP-3 expression and secretion.²⁹

Mechanistic studies have suggested that the RPE/BrM/choroid interface is the target of prolonged, low grade, predominantly sublytic complement activation in AMD. We have previously developed a culture model of sublytic complement activation in RPE monolayers, which requires the exposure of cells to oxidative stress in the presence of complement sufficient normal human serum (NHS).²⁶ Here, we tested the hypothesis that complement activation on RPE monolayers alters MMP secretion, and by increasing MMP activity levels, changes the availability of angiogenic factors in the extracellular space. Complement activation on primary human RPE monolayers was sublytic in nature and did not alter cellular integrity or intracellular adenosine triphosphate (ATP) levels. However, the transient activation increased MMP-2 and

MMP-9 expression and activation in the extracellular space. Since MMP activity was found to mobilize vascular endothelial growth factor (VEGF) and degrade pigment epithelium-derived factor (PEDF), as a net result, the ratio of VEGF/PEDF was altered significantly, shifting the balance to a proangiogenic state.

METHODS

Human Fetal RPE Cell Culture

Human fetal RPE cells were prepared following the protocol by Maminishkis et al.³⁷ with some modifications. Human donor eyes were used in accordance with the provisions of the Declaration of Helsinki for research involving human tissue. Briefly, fetal eyes (in transportation buffer) were supplied by Advanced Bioscience Resources (Alameda, CA). Upon arrival, eyes were washed with Hanks' balanced salt solution (HBSS; Invitrogen, Carlsbad, CA), excessive tissues were removed, and the anterior chamber and vitreous were discarded. The posterior pole was incubated in Accutase solution (Invitrogen) for 10 to 20 minutes. Eyes were then transferred to fresh HBSS where the retina was removed and the RPE sheet carefully separated from the choroid. RPE sheets were collected in cold minimum essential medium (Sigma-Aldrich, St. Louis, MO) with 5% fetal bovine serum (FBS; Invitrogen). After centrifugation, medium was removed, and the RPE sheets were incubated in trypsin solution for 10 minutes followed by gentle pipetting to obtain a cell suspension. Cells were then transferred to a flask with medium containing 15% serum and cultured in a humidified incubator at 37°C, in 5% CO₂ and 95% air. After 2 days of culture, 15% serum was replaced by 5% serum for 2 to 3 weeks until they formed a monolayer and showed pigmentation.

RPE Monolayer

Cells (2×10^5) were plated on Transwell plates (0.4 μ m Polyethylene Terephthalate [PET], 24-mm insert; Corning, Corning, NY) and cultured for another 2 to 3 weeks until they showed stable transepithelial resistance (TER), as measured using a STX2 electrode (volt-ohm-meter; World Precision Instruments, Sarasota, FL). Resistance values were corrected for background resistance produced by the insert in the presence of medium. RPE cells form tight monolayers, which are polarized and develop tight and adherence junctions. Confluent monolayers with stable TER were used for experiments. Monolayers were kept in serum-free medium 24 hours prior to each experiment. The apical and basal mediums were replaced with fresh serum-free medium before experimentation (2 mL each). Monolayers were treated with 0.5 mM H₂O₂, 25% NHS, or H₂O₂ + NHS in combination. At the end of the experiments, supernatants from both the apical and basal sides as well as the monolayers were collected for further study.

Reagents

The reagents used in these studies included pooled NHS (Quidel, Santa Clara, CA) as a source of complement proteins. To prevent formation of the terminal complement pathway, C7-depleted serum (Quidel) was used, and purified C7 (250 μ g/mL; Quidel) was added to the serum in some experiments. To block the AP of complement activation, a targeted inhibitory protein (CR2-fH; 10 μ g/mL) was produced as previously described.³⁸ This agent targets the inhibitory domain of factor H to sites of C3d deposition and effectively blocks AP activation. The VEGFR-1/2 inhibitor, 10 μ g/mL SU5416 (Chemicon, Millipore, Billerica, MA), was used to block the effects of VEGF SU5416 (Z-3-[(2,4-dimethylpyrrol-5-yl) methylidene]-2-indolinone) is a lipophilic synthetic receptor tyrosine kinase inhibitor, which inhibits VEGFR-1/2 by binding to the ATP binding pocket within the kinase domain of the receptor. SU5416 has been shown to inhibit VEGF-dependent endothelial cell proliferation in vitro and in animal models.³⁹ To inhibit

the activity of MMP-2 and MMP-9, a potent inhibitor specific for those two enzymes only, BiPS (2R)-[(4-Biphenylsulfonyl)amino]-N-hydroxy-3-phenylpropionamide (EMD Biosciences, Gibbstown, NJ), was used at 10 μ M concentration.

Immunocytochemistry

RPE monolayers were fixed in 4% paraformaldehyde, washed in PBS, and blocked with 10% normal goat serum (Jackson ImmunoResearch Laboratories, West Grove, PA) and 3% bovine serum albumin in PBS. Monolayers were incubated in blocking solution containing the antibodies of interest for 2 hours, followed by incubation with the appropriate fluorescent-labeled secondary antibody for 1 hour (Molecular Probes, Carlsbad, CA). Monolayers were mounted and analyzed by confocal microscopy (Leica, Bannockburn, IL) using identical settings for all slides. The following primary antibodies were used in this study: Rabbit anti-Zo-1 (Zymed, San Francisco, CA) and rabbit antioccludin (Zymed).

Enzyme-Linked Immunosorbant Assays (ELISAs)

After each experiment, media were collected from apical and basal compartments of the Transwell plate. VEGF concentrations were assayed using the protocol provided by the manufacturer of the human VEGF ELISA kit (Antigenix America, Inc., Huntington Station, NY); PEDF concentrations were determined according to the protocol supplied with the human PEDF ELISA kit (Bioproducts MD, Middletown, MD).

Quantitative Real-Time PCR (QRT-PCR)

QRT-PCR was performed as previously described.⁴⁰ In short, RNA (2 μ g each) was used to generate cDNA in reverse transcription reactions (Invitrogen). PCR amplifications were conducted using the QuantiTect Syber Green PCR Kit (Qiagen, Valencia, CA) using the following primers: β -actin, 5'-AAA TCT GGC ACC ACA CCT TC-3', reverse 5'-GGG GTG TTG AAG GTC TCA AA-3'; VEGF, forward 5'-TCT TCA AGC CAT CCT GTG TG-3', reverse 5'-ATC CGC ATA ATC TGC ATG GT-3'; PEDF, forward 5'-CTG CAG GGA CTT GGT GAC TT-3', reverse 5'-GTC GGA CCC TAA GGC TGT TT-3'; MMP2, forward 5'-ATG ACA GCT GCA CCA ATG AG-3', reverse 5'-AGT TCC CAC CAA CAG TGG AC-3'; and MMP9, forward 5'-CAT CGT CAT CCA GTT TGG TG-3', reverse 5'-AGG GAC CAC AAC TCG TCA TC-3'. Quantitative values were obtained from the cycle number (Ct value) to establish the fold-difference in gene expression between the treated and untreated RPE cell monolayers.⁴⁰

Quantification of ATP

ATP level was measured by following the Celltiter-Glo luminescent cell viability assay protocol (Promega, Madison, WI). According to this protocol, the generation of a luminescent signal is proportional to the amount of ATP present, and the amount of ATP is directly proportional to the number of metabolically active cells present in the culture. Luminescence was recorded using an integration time of 0.25 seconds per well.

Western Blot Analysis

Cell culture supernatants were separated by electrophoresis on a 10% BisTris polyacrylamide gel (Invitrogen), and proteins were transferred to a nitrocellulose membrane. Membranes were probed with polyclonal antibodies to MMP-2 (IM33; EMD Biosciences) and MMP-9 (ab38898; Abcam, Cambridge, MA) overnight in LI-COR Odyssey blocking buffer (LI-COR Biosciences, Lincoln, NE). After washing, membranes were incubated with their respective secondary antibodies for 1 hour. After washing, the immunoreactive membrane bands were detected by using an appropriate scanner (LI-COR) and the density of each band was analyzed by using Image J software (free software developed by the National Institutes of Health).

Zymography

MMP-2 and MMP-9 activity was assayed by using the Novex zymography protocol (Invitrogen). For gelatin zymographs, equal volumes of supernatants were loaded in 10% Novex zymogram gels diluted with sample buffer (2 $\times$). After electrophoresis, gels were incubated in renaturing buffer for 30 minutes at room temperature, equilibrated in Novex zymogram developing buffer for 30 minutes, and incubated overnight at 37°C. Gels were washed with water and stained with Coomassie blue. Excess stain was removed by washing with water. Gels were scanned, and density analyses of the bands were performed by using Image J software.

Intracellular Reactive Oxygen Species (ROS) and Superoxide Assays

Intracellular ROS and superoxide ($O_2^{\cdot -}$) levels were measured using 2',7'-dichlorofluorescein diacetate dye and dihydroethidium, respectively, following the manufacturer's protocol (Molecular Probes/Invitrogen). Intracellular ROS and superoxide levels were assessed by monitoring fluorescence levels (Softmax Pro, version 4.8, Molecular Devices, Sunnyvale, CA) at an excitation/emission of 480/520 nm and 530/590 nm, respectively.⁴¹

RESULTS

Complement Activation in RPE Monolayers Results in Reduction in Barrier Function

Primary human embryonic RPE cells were grown on Transwell plates. Primary RPE monolayers grown on these porous supports have been shown to exhibit morphology and physiology of native tissue,³⁷ including the generation of extracellular deposits.⁴² Monolayer formation was monitored using TER measurements, which is a convenient readout to probe for a breach of barrier function integrity as an early marker of RPE injury.^{26,43} After the monolayers achieved a stable TER level of 200 to 300 ohms/cm², typically within 2 to 3 weeks of reaching confluence, FBS was removed from the growth media, which had no effect on the TER of established monolayers.^{26,43} Monolayers could then be treated with apical application of 0.5 mM H_2O_2 to induce oxidative stress, 25% NHS (as a source of complement proteins), or both (Fig. 1). Four-hour treatment of the monolayers with either 0.5 mM H_2O_2 or NHS alone had little effect on TER. However, the combined treatment of H_2O_2 + NHS significantly decreased TER ($23.03 \pm 1.2\%$) when compared to the untreated control; heat-inactivated NHS, however, was ineffective. To confirm that this effect was in part due to sublytic complement activation rather than some other heat-labile component present in NHS, H_2O_2 -exposed cells were treated with C7-depleted serum. C7-depleted serum was significantly less effective in reducing TER ($P < 0.01$), whereas the addition of purified C7 partially restored the effect. To confirm that the sublytic MAC effect on TER requires the involvement of the complement AP, monolayers were pretreated with CR2-fH, a potent, targeted form of complement factor H,²⁴ which significantly blunted the effect of H_2O_2 + NHS. Since barrier function in the RPE is controlled by VEGFR2 receptors on the apical surface of the RPE,⁴³ and complement activation controls the secretion of VEGF protein from ARPE-19 cells,²⁷ we examined the effects of H_2O_2 + NHS in the presence of SU5416, a potent VEGFR1/2 inhibitor. Pretreatment of the monolayers with SU5416 prevented the deterioration of TER in response to complement activation.

Thus far we have shown that exposure of H_2O_2 -treated primary RPE cell monolayers to NHS impaired their barrier

FIGURE 1. TER in human primary RPE cell monolayers was susceptible to sublytic complement attack. RPE cells were grown until a stable TER was obtained. Monolayers were treated with 0.5 mM H₂O₂, 25% NHS, or H₂O₂ + NHS in combination. Treatment with H₂O₂ or NHS alone caused a slight drop in TER, whereas combined treatment with H₂O₂ + NHS caused a significant decline in TER after 4 hours of treatment time. Monolayers treated with H₂O₂ and heat-inactivated (HI) serum, were unaffected. C7-depleted serum was significantly less effective in reducing TER, whereas the addition of purified C7 restored the effect. Prior administration of CR2-fH (an AP inhibitor²⁶) prevented the decline in TER, as did the addition of a VEGF-receptor-1/2 antagonist (SU5416). Data are expressed as mean $\pm$ SEM ($n = 3-6$). Statistical comparisons accept a $P < 0.01$ (*).

function. The requirement of complement component C7 together with a lack of cell death²⁶ classifies this paradigm as sublytic complement activation. Oxidative stress was confirmed by quantifying cytosolic ROS generation and super oxide production (O₂⁻) with dichlorofluorescein diacetate and dihydroethidium, respectively (Figs. 2A, 2B). ROS levels increased significantly in the H₂O₂ + NHS-treated monolayers when compared to the other three sets ($P < 0.05$). On the other hand, O₂⁻ levels in NHS versus H₂O₂ + NHS-treated monolayers were indistinguishable. Lack of cytotoxic effect was confirmed by monolayer morphology (Fig. 2C). RPE monolayers were stained with antibodies against ZO-1 and occludin to study tight junction integrity. No differences in labeling could be identified in cells exposed to H₂O₂ + NHS when compared to controls. Finally, increased ROS production, as well as the presence of MAC pores in the cell membrane, could lead to the loss of intracellular ATP.⁴⁴ Intracellular ATP concentration, however, did not differ across the four different groups (control, H₂O₂ alone, NHS alone, and H₂O₂ + NHS [Fig. 2D]). Thus, while complement exposure of oxidatively stressed cells increased ROS production and impaired the barrier function of the cellular monolayer, in this system the drop in TER was not due to direct cytotoxicity.

Complement Activation in RPE Monolayers Resulted in an Increase in MMP-2 and MMP-9 Secretion and Protease Activity

MMP-2/9 expression has been found to have increased activity in AMD (e.g., Ref. 34). However, neither the mechanism for MMP expression and activation nor the targets for its proteolytic activity have been examined in models of AMD.

FIGURE 2. Effect of sublytic complement activation on cellular integrity. Intracellular ROS (A) and super oxide production (B) were measured using dichlorofluorescein diacetate dye and dihydroethidium, respectively. ROS levels were significantly elevated in H₂O₂ + NHS-treated cells when compared to single treatments (H₂O₂ or NHS), whereas superoxide levels did not show any significant change between the treatment groups, although NHS and H₂O₂ + NHS treatment groups had elevated superoxide levels when compared to control cells. Data are expressed as mean $\pm$ SEM ($n = 3-4$ per condition). (C) RPE monolayers were stained with antibodies against ZO-1 and occludin to confirm that H₂O₂ + NHS treatment did not affect structural integrity of tight junctions at the light-microscopy level in RPE monolayers when compared to controls. (D) Although MAC is permeable to small molecules, including ATP, overall ATP levels did not differ among all experimental conditions when tested 4 hours after the treatment.

To determine the effect of oxidative stress and complement activation on MMP-2/9 secretion, supernatants of human primary RPE monolayer cultures were analyzed by Western blotting, using specific antibodies. NHS was found to contain significant amounts of MMP-2 and MMP-9, such that potential apical secretion could not be analyzed. However, apical H_2O_2 + NHS increased significantly the levels of basal MMP-2 and MMP-9 present in the supernatant; while H_2O_2 or NHS alone had little effect (Fig. 3A). MMP activity, as assessed by gelatin zymography followed by densitometry of the bands, revealed that both MMP-2 ($P < 0.05$) and MMP-9 ($P < 0.01$) activity levels were increased significantly in the basal supernatant of H_2O_2 + NHS-treated RPE monolayers when compared to the untreated ones (Fig. 3B). This increase in MMP activity was complement component C7-dependent; MMP-2/9 activity levels were significantly reduced in monolayers treated with H_2O_2 + C7-depleted sera, whereas the addition of purified C7 protein reinstated the effect (data not shown).

In addition to increasing the level of activated MMP-2/9 in the supernatant, complement activation increased mRNA production for both enzymes as measured by QRT-PCR. H_2O_2 + NHS cotreatment increased levels significantly over treatment with either H_2O_2 or NHS (Fig. 3C).

MMP Activity Affects the Balance of VEGF/PEDF

Since our results suggested that apical complement activation by H_2O_2 + NHS resulted in TER reduction mediated by VEGF (see SU5416 effects in Fig. 1), and MMP-2/9 was reported to mobilize matrix-bound VEGF³⁵ and degrade PEDF³⁶ we examined TER as well as VEGF and PEDF levels in the RPE monolayer supernatants in the presence and absence of a potent MMP-2/9 inhibitor, BiPS. TER was reduced when using a 2-hour H_2O_2 + NHS treatment, an effect that was significantly blunted when BiPS was coadministered. BiPS alone had no effect on TER (Fig. 4A). VEGF levels were not altered by H_2O_2 or NHS when applied individually to the apical chamber. However, coadministering H_2O_2 + NHS to the apical surface resulted in ~30-fold increase in apical VEGF levels, and an ~5-fold increase in basal levels (Fig. 4B). On the other hand, levels of the antiangiogenic factor PEDF were not increased by complement activation (Fig. 4C). Thus, in the presence of complement and oxidative stress, the balance of VEGF/PEDF is tilted towards VEGF. In the presence of BiPS, H_2O_2 + NHS, this ratio is tilted even further towards a proangiogenic state. When the MMP inhibitor, BiPS, is added together with H_2O_2 + NHS to either the apical or basal supernatant, VEGF levels were significantly decreased in the supernatant (Fig. 4B). Conversely, MMP inhibition resulted in a significant increase in PEDF in the apical and basal supernatants (Fig. 4C). The addition of BiPS to H_2O_2 + NHS-treated cells did not alter mRNA expression levels of either of the two growth factors (Fig. 4D), neither did BiPS alone have any effect(s) on growth factor mRNA (Fig. 4D) nor protein levels (Figs. 4B, 4C). Overall, the amount of VEGF in the apical supernatant correlated with the amount of TER reduction ($R = 0.92$, $P < 0.0001$; Fig. 4E). These observations suggested that MMP activity is involved in VEGF mobilization from protease-accessible binding sites on both the apical and basal membrane of the RPE cell monolayer, as well as in PEDF degradation on both sides of the monolayer.

DISCUSSION

The main results of the current study are: (1) sublytic complement activation resulted in the impairment of barrier function in primary RPE cell monolayers; (2) reduction in

A [Protein]

B [Activity]

C [mRNA]

FIGURE 3. Sublytic complement activation resulted in MMP activation in primary RPE cells. Primary RPE cells were grown as described in the text. Supernatant and cells were collected after 4 hours for (A) Western blot analysis (protein), (B) zymography (activity), and (C) QRT-PCR (mRNA). Data are expressed as mean $\pm$ SEM ($n = 3$ –6 per condition). (A) MMP-2 and MMP-9 secretion into the basal compartment as examined by Western blotting was found to be significantly increased. Due to the presence of MMPs in the serum, only the basal levels were measured. (B) MMP-2 and MMP-9 activity levels were assessed by gelatin zymography followed by densitometry of the bands. The band representing activated MMP-2 and MMP-9 run at 68 and 88 kDa, respectively. Both MMP-2 and MMP-9 activity levels were increased significantly in the basal supernatant of H_2O_2 + NHS-treated RPE monolayers, when compared to the untreated monolayers. (C) MMP-2 and MMP-9 mRNA expression levels were also increased after 4 hours of treatment as determined by QRT-PCR, using β -actin as the control.

barrier function was associated with the secretion of VEGF, a growth factor that has been shown to disrupt RPE barrier function⁴³; (3) complement activation, even though it resulted in the generation of ROS, did not impair cellular integrity or result in a loss of ATP; (4) complement activation increased MMP-2/9 expression and resulted in the release of these

FIGURE 4. Sublytic complement activation and MMPs decrease the VEGF/PEDF ratio in primary RPE cell monolayers. Primary RPE cells were grown as stable monolayers. TER was measured, and supernatants and cells were collected for further analysis. Data are expressed as mean $\pm$ SEM ($n = 3-6$ per condition). Statistical comparisons accept a $P < 0.05$. * $P < 0.05$; ** $P < 0.01$. (A) Treatment with H₂O₂ + NHS caused a significant decline in TER after 2 hours of treatment time, whereas monolayers treated with H₂O₂ + NHS in the presence of the

enzymes into the supernatant, as well as their activation; (5) activated MMP-2/9 were found to play a significant role in altering the VEGF/PEDF balance on the apical and basal sides of the RPE monolayer; and (6) MMP activity was found to increase VEGF, and to decrease PEDF levels without altering their respective expression levels. Thus, oxidative stress and the presence of complement together generated a cellular environment that promotes AMD pathology.

The involvement of complement activation in the pathogenesis of AMD is now well recognized, which is also reflected in the significant effort to develop complement therapeutics for the treatment of AMD.⁴⁵ However, it is still unclear which activation products of the complement cascade participate in pathogenesis. The comparison of effective complement therapeutics in mouse models with the presence of complement activation components does not, unfortunately, further clarify the problem. In the mouse model of wet AMD, CNV can be ameliorated by targeting the anaphylatoxin receptors,⁴⁶ the MAC,⁴⁷⁻⁵⁰ or the AP convertase.²⁴ In patients with AMD, drusen and basolaminar deposits are immunopositive for C3a, C5a,⁴⁶ cell-bound complement component C3 activation fragments, and MAC proteins,^{13,16-18} as well as CFH.¹⁹ Since RPE-damage seems to be involved in all forms of AMD, and the RPE might be the source of many of the proteins present in drusen,⁵¹ our focus has been on complement activation at the level of the RPE. Complement proteins can be divided into three classes: receptors, that interact with complement activation products generated during complement activation; regulatory proteins that limit complement activation; and components that generate the effector function of complement. Protein expression of complement components required to allow for cell-specific complement activation, such as the expression of the anaphylatoxin receptors, C3aR and C5aR, has been documented on RPE cells,⁵² as has the expression of membrane-bound complement inhibitors (CD46, CD55, and CD59^{26,53}) and the regulated secretion of factor H.⁵⁴ On the other hand, complement components required for complement activation, belonging to either the CP, LP, AP, or the common terminal pathway, are primarily synthesized by the liver hepatocytes and circulated in the blood as inactive precursors until they reach an activatable surface. The combined results of DNA microarray and QRT-PCR studies on

MMP inhibitor BiPS were unaffected. (B) Apical and basal VEGF levels were determined by an ELISA specific for human VEGF. RPE monolayers were treated with 0.5 mM H₂O₂, 25% NHS, or H₂O₂ + NHS in combination; supernatants were harvested after 2 hours of incubation and VEGF levels determined by ELISA measurements. Two hours of sublytic MAC activation was found to increase VEGF levels on the apical side of the RPE monolayer by ~ 30 -fold, and ~ 4 -fold on the basal side. Pretreatment of the monolayer with BiPS, an MMP-2/9 inhibitor, prior to H₂O₂ + NHS exposure, reduced the VEGF level significantly in both the apical and the basal compartments. BiPS alone had no effect when compared to controls. A serum control (NHS without the exposure to cells) was used to examine VEGF levels present in NHS. (C) Apical and basal PEDF levels were determined by an ELISA specific for human PEDF. Apical and basal PEDF levels were only marginally increased by sublytic complement activation when compared to H₂O₂ or NHS cells, whereas the levels were elevated significantly in the basal supernatant when compared to untreated cells. Pretreatment of cells exposed to H₂O₂ + NHS with BiPS significantly increased the PEDF level on both sides. BiPS alone had no effect when compared to controls. A serum control (NHS without the exposure to cells) was used to examine PEDF levels present in NHS. (D) VEGF and PEDF mRNA expression levels were unaltered after 2 hours of treatment as determined by QRT-PCR, using β -actin as the control. (E) When apical VEGF concentration was plotted against the percentage reduction in TER, correlation was observed ($R = 0.92$, $P < 0.0001$).

human RPE and choroid suggest that while at the mRNA level, some of the components required for complement activation are expressed at low levels in the RPE⁵⁵⁻⁵⁸, the main source of complement proteins appears to be the choroid plexus.⁵⁹ However, even if the resulting complement protein concentrations were to be high enough to support a complement attack, their respective serum concentrations range from tens of microgram to low milligram per milliliter,⁶⁰ activation would be limited to the AP and CP branches, since most of the components for the LP and the common terminal pathway appear to be missing in the choroid.⁵⁹ Thus, it remains to be determined whether complement activation is driven at the level of the RPE solely by systemically delivered complement components or whether locally derived components might contribute to the effect. Complement activation will occur due to an imbalance of complement activators and inhibitors. Our earlier observations have demonstrated that oxidative stress, generated by exposing RPE monolayers to nonlethal amounts of H₂O₂, resulted in the reduction of membrane-bound CD55 and CD59, and altered the RPE cells in such a way that factor H is less functionally protective.²⁶ Interestingly, in geographic atrophy, a condition thought to be associated with prolonged oxidative stress, CD46 is decreased, which similarly might increase the risk for complement injury.⁶¹ Similar data on complement inhibitor levels from patient samples with other forms of AMD are currently missing, and the functional analysis of the protective (Y402) and the risk form (402H) of factor H are inconclusive.⁶²⁻⁶⁴

Since RPE cell death does not typically occur until later in the progression of the disease, we have focused our attention on sublytic activation of the complement cascade, in particular since sublytic activation affects cell behavior that might be important in AMD such as secretion, adherence, or membrane function (reviewed in Ref. 8). Sublytic activation is thought to result in transient MAC pore formation⁶⁵; although we have not yet demonstrated the presence of MAC pores in human primary RPE or ARPE-19 cells. MAC pores consist of a tetramolecular C5b-8 ring- and poly-C9 tubular-complex, with the diameter of the pore being controlled by the number of C9 molecules, requiring >12 molecules of C9 for discrete channel formation.⁶⁶ Pore formation would allow free movement of small molecules in and out of the cell. Three molecules known to penetrate the pores are of particular interest for this work: MAC activation can result in ATP efflux and Ca²⁺/Na⁺ influx.⁶⁷ The data presented here indicate that ATP loss does not occur under our stimulation conditions. Intracellular ATP levels remained stable over the 4-hour examination period; although the possibility of increased ATP synthesis in the presence of ATP loss through the presumed MAC pores was not examined. Our previous data on ARPE-19 cells would suggest that complement activation in a complement component C7-dependent manner triggers Ca²⁺-activated pathways (Erk, Ras, and Src)²⁷ and depolarizes the cell membrane (Rohrer B, et al. *IOVS* 2011;52:E-Abstract 2316). Both mechanisms could modulate secretory pathways in RPE cells.

Here, we investigated whether complement activation alters levels of MMPs and angiogenic factors in supernatants of primary human RPE cells. In particular, we examined the involvement of MMP-2/9 based on the known functions of these two enzymes in processing growth factors and focused on the two main factors known to modulate barrier function in the RPE the proangiogenic factor, VEGF, and the antiangiogenic factor, PEDF.^{43,68} MMP-2/9 mRNA expression in primary RPE cells was significantly increased after 4 hours of treatment with H₂O₂ + NHS, whereas supplying either H₂O₂ or NHS had no effect. In addition, H₂O₂ + NHS treatment resulted in elevated levels of MMP-2/9 protein in the supernatant, and activated MMP-2 and MMP-9 could be documented by gelatin zymog-

raphy and by inhibitor assays. MMP-2/9 activity was dependent upon sublytic complement activation, since levels were significantly reduced in monolayers treated with H₂O₂ + C7-depleted sera and reconstituted when purified C7 protein was added back. The main focus for MMP analysis in AMD has been on ECM remodeling, since MMP-2 and MMP-9 degrade basement membrane components such as type IV collagen present in BrM (e.g., Ref. 34). However, here we have focused on the second set of targets for MMP activity: growth factors. Previous reports have shown that, for example in tumor growth, MMP activity is involved in mobilizing matrix-bound VEGF, thus promoting angiogenesis³⁵; whereas in RPE conditioned media, activated MMP-2 or MMP-9 can degrade PEDF.³⁶ Our data support these observations, since inhibiting MMP-2/9 activity with BiPS, a unique inhibitor that only suppresses MMP-2/9 activity, in the presence of H₂O₂ + NHS resulted in a decrease in VEGF levels and an increase in PEDF levels present on both the apical and basal sides of the RPE cell monolayer. MMP-2/9 inhibition in the presence of H₂O₂ + NHS, however, had no effect on VEGF or PEDF expression levels. The inference from these results is that MMP-2/9 activity might mobilize VEGF by releasing it from its ECM-binding sites,³⁵ whereas PEDF might be a substrate for MMP-mediated proteolytic cleavage.³⁶ Alternatively, MMP-2/9 activity might stimulate VEGF and inhibit PEDF release from RPE cells or activate other factors involved in the modulation of the release of these two factors. Irrespective of the mechanism, as a net result, MMP-2/9 activity increases the VEGF/PEDF ratio, shifting it towards angiogenesis. Important in this context is that BiPS alone, in the absence of oxidative stress and serum, had no effect on growth factor levels. BiPS has recently been found to have off-target effects on Hif1 α levels, preventing its degradation,⁶⁹ and the VEGF gene is a target for the transcription factor, Hif1 α .⁷⁰ An off-target effect through modulating Hif1 α levels would have independently led to an increase in VEGF, which was, however, not observed under our experimental conditions. Dysregulation of the VEGF/PEDF ratio in RPE cells has been reported previously. Pons and Marín-Castaño⁷¹ have shown that cigarette smoke-related hydroquinone treatment dysregulates the VEGF/PEDF ratio in RPE in vitro (ARPE-19 cells) and in vivo (mouse), and that RPE cells isolated from smokers have an increased VEGF/PEDF ratio. However, the possibility that factors other than oxidative stress might be involved in creating this imbalance in VEGF and PEDF was not addressed by these authors. Finally, while increased VEGF levels are known to be associated with AMD, a direct correlation between the amount of VEGF production and disease severity is lacking. Here, we demonstrated a direct and highly significant correlation ($P < 0.0001$) between VEGF levels in the apical supernatant and the reduction of barrier properties in the RPE monolayers.

In conclusion, we have added evidence that oxidative stress and the presence of complement components together generate a cellular environment that promotes AMD pathology. Our findings provide a mechanism whereby complement activation of RPE cells results in increased levels of a number of molecules implicated in the pathogenesis of AMD, including VEGF and MMPs. Once released into the supernatant and activated, the MMPs mobilize VEGF from heparin-binding sites and degrade PEDF, skewing the ratio of these two growth factors toward an angiogenic profile. Our results, therefore, add yet another event in the development of AMD that occurs at the level of the RPE, is controlled by complement, and participates in creating the observed pathologic VEGF levels. Therapeutic complement inhibitors may, therefore, prevent the formation of several pathogenic factors and slow the progression of AMD.

Acknowledgements

We thank Luanna Bartholomew for critical review (Medical University of South Carolina, Charleston, SC).

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