Nitrite Modification of Extracellular Matrix Alters CD46 Expression and VEGF Release in Human Retinal Pigment Epithelium

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PURPOSE. Loss of CD46 has recently been implicated in choroidal neovascularization in mice. Herein we investigated the effect of nitrite modification of the extracellular matrix (ECM) as an in vitro model of "aging" and its effect on CD46 expression and vascular endothelial growth factor (VEGF) release in cocultured human retinal pigment epithelium (RPE).

METHODS. ARPE-19 cells were plated onto RPE-derived ECM conditions (untreated; nitrite modified; nitrite modified followed by washing with Triton X-100; or nitrite modified followed by washing with Triton X-100 and coated with extracellular matrix ligands). Cells were cultured for 7 days and CD46 expression was analyzed by immunohistochemistry and Western blot. Additionally, CD46 short interfering RNA (siRNA) was transfected into ARPE-19 cells, and VEGF levels were determined by ELISA. Finally, in the same ECM conditions, ARPE-19 cells were challenged with normal human serum and VEGF levels determined by ELISA.

RESULTS. CD46 is expressed on the basolateral surface of ARPE-19 cells on RPE-derived ECM. Nitrite modification of ECM reduced the expression of CD46 on ARPE-19 cells by 0.5-fold (P < 0.003) and increased VEGF release in ARPE-19 cells by 1.7-fold (P < 0.001). CD46 knockdown also increased release of VEGF on the apical and basal sides of ARPE-19 cells in culture by 1.5- (P = 0.012) and 1.2-fold (P = 0.017), respectively.

CONCLUSIONS. Nitrite modification of the ECM decreased CD46 expression and increased the release of VEGF from ARPE-19 cells. Changes in CD46 expression may lead to changes in VEGF and play a pathologic role in the development of age-related macular degeneration.

Keywords: age-related macular degeneration, retinal pigment epithelium, CD46, vascular endothelial growth factor, Bruch’s membrane

Age-related macular degeneration (AMD) is a leading cause of blindness in the United States and Western Europe.1 Among patients older than 70 years, 30% to 50% will have some evidence of this disease, and severe loss of vision is present in 2%. Loss of vision in the setting of advanced AMD results from either choroidal neovascularization (CNV; “exudative” or “wet” AMD) or geographic atrophy (GA). Geographic atrophy involves a well-defined area of loss of retinal pigment epithelium (RPE), underlying choriocapillaris, and thinning of the overlying neurosensory retina.2 The precise mechanisms underlying both AMD phenotypes are not well understood.

Age-related damage to RPE is associated with changes to Bruch’s membrane (BM), which serves as a substrate for RPE attachment.3 Age-related changes in BM include diffuse thickening, accumulation of drusen, basal laminar deposits, basal linear deposits,4,5 collagen cross-linking in the inner and outer collagen layer, calcification and fragmentation of the elastin layer,6 and lipidization.6–8 These changes precede RPE cellular changes and dysfunction by 1 to 2 decades in AMD patients.8,9 Age-related changes in human BM induce multiple deleterious effects on RPE function, including cell adhesion, proliferation, differentiation, migration, and the ability to phagocytize outer segments.3,4,7,9,10 Chronic inflammation, which is strongly associated with AMD, contributes to these structural changes in BM and can have additional deleterious effects on RPE fate, thus leading to RPE loss and eventual atrophy.5,11–16

The production of nitric oxide, a cross-linking agent, has been implicated in chronic inflammation and the production of its byproducts such as nitrite, thus subjecting these areas to increased nitrosative stress. Another contributor to chronic nitric oxide production and subsequent nitrite exposure is cigarette smoking, a risk factor strongly associated with AMD.17,18 These changes contribute to the cumulative damage to the BM and result in the age-related collagen cross linking, a
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**Preparation of ECM**

To prepare the RPE ECM nitrite-treated plates, we used a method previously described. Briefly, ARPE-19 cells were grown on 24-well Transwell permeable supports (Corning, Tewksbury, MA, USA) in 12-well plates for 6 to 8 weeks to allow for ECM formation. ARPE-19 cells were removed by the addition of 20 mM ammonium hydroxide buffer for 20 minutes, and the ECM was washed three times with phosphate-buffered saline (PBS). Phosphate-buffered saline was removed from the RPE-derived ECM (RPE-ECM), which was then allowed to dry. The RPE-derived ECM plates were either used immediately after drying, or stored at −20°C for future use. RPE-derived ECM was processed further to create four experimental plating surfaces (untreated, nitrite modified, nitrite modified followed by washing, or nitrite modified followed by washing and coating with a mixture of ECM ligands).

**Nitrite Modification**

Nitrite-modified ECM was prepared by adding 100 mM sodium nitrite to the ECM and incubating at 37°C for 7 days. Plates were then washed at least four times with PBS and further incubated with PBS for 4 hours. Following this, the plates were washed at least two additional times to completely remove the nitrite.

**Extracellular Matrix Detergent Cleaning.** Triplicate wells were treated with 0.1% Triton X-100/0.1% sodium citrate solution for 20 minutes at 4°C. After cleaning, the ECM was washed three times with PBS for 5 minutes and prepared for plating of ARPE-19 cells.

**Addition of ECM Proteins.** All ECM in this treatment condition was detergent cleaned as described above. Following this, triplicate wells were incubated with an ECM–protein mixture containing laminin (330 μg/mL; Sigma-Aldrich Corp., St. Louis, MO, USA), fibronectin (250 μg/mL; Sigma-Aldrich Corp.), and vitronectin (35 μg/mL; Corning Incorporated Life Sciences, Tewksbury, MA, USA) at 37°C for 30 minutes. The ECM was then washed three times with PBS for 5 minutes and prepared for plating of ARPE-19 cells.

**Immunohistochemistry**

Cell cultures were fixed with 4% paraformaldehyde at 4°C for 15 minutes, then permeabilized by incubation for 5 minutes in 0.1% Triton X-100 in PBS and incubated with 0.1% bovine serum albumin and 1% normal goat serum in PBS for 45 minutes. Cells were incubated with a rabbit polyclonal antibody to CD46 (Invitrogen-Gibco, Life Technologies) and a mouse monoclonal antibody to zonula occludens-1 (ZO-1; Invitrogen-Gibco, Life Technologies) overnight at 4°C. Cells were washed and incubated for 1 hour at 37°C in the dark with a goat anti-rabbit IgG antibody conjugated to Alexa Fluor 594 (red fluorescence; Invitrogen-Molecular Probes, Grand Island, NY, USA) and a goat anti-mouse IgG antibody conjugated to Alexa Fluor 488 (green fluorescence; Invitrogen-Molecular Probes). Nuclei were stained with 4′,6-diamidino-2-phenylindole (DAPI) by incubating the cells in the dye solution for 10 minutes. The cells were then washed four times in PBS. Immunologically-stained cell cultures were visualized by a Zeiss LSM 510 NLO (Zeiss, Jena, Germany) confocal laser scanning microscope (Medical University of South Carolina Cell and Molecular Imaging Shared Resource) using a Plan-Apochromat 63X/1.4 oil DIC objective (Zeiss).

**Western Blot Analyses of CD46 Expression**

To detect expression of CD46 in ARPE-19 cells, cell suspensions were lysed and separated by electrophoresis on a 10%...
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CD46 Silencer siRNA Transfection

ARPE-19 cells were cultured on 24-well inserts and grown to confluence for 7 days. CD46 or random siRNA were transfected into confluent ARPE-19 cells. Amine transfection agent (Ambion, Life Technologies, Grand Island, NY, USA) was used to transfect the siRNA oligos (Ambion, Life Technologies) into cultured ARPE-19 cells. Culture media from the basal (in 24-well plates) or apical (within inserts) side of ARPE-19 cultures was collected 48 hours post transfection. CD46 expression was determined by Western blot as described above, and VEGF was determined by ELISA using a human VEGF Quantikine ELISA Kit (R&D Systems, Minneapolis, MN, USA), and results were analyzed by using a FLx 800 Microplate Reader (Biotek, Winooski, VT, USA) at 1 week. The resistance values (Ω·cm²) for the monolayers were determined from the average of four independent measurements, and resistance values were corrected for background resistance produced by the insert in the absence of RPE-derived ECM and medium. The resistance values for all groups ranged between 51 and 66 Ω·cm², which are in the range of resistance for confluent ARPE-19 monolayers.

Data Analyses

One-way ANOVA and t-tests were performed with Prism (GraphPad Software, Inc., La Jolla, CA, USA). A criterion of α = 0.05 was adopted. Each experiment was done in triplicate.

RESULTS

CD46 Is Expressed Basolaterally in ARPE-19 Cells on RPE-ECM

Confocal microscopy revealed positive ZO-1 expression at the cell margins of ARPE-19 cells (apical intercellular area) seeded on RPE-ECM (Fig. 1A). CD46 staining was present intracellularly and at the basal margins of the cell (B), DAPI nuclear staining (C), and merged image (D). XZ projection shows apical expression of ZO-1 and basolateral and intracellular localization of CD46 (E). Scale bars: 20 μm. Green = anti-ZO-1, red = anti-CD46, blue = DAPI. The density of the protein bands was accomplished by measuring band intensity with ImageJ software (http://imagej.nih.gov/ij/; provided in the public domain by the National Institutes of Health, Bethesda, MD, USA); CD46 data were normalized to the total GAPDH control.

Extracellular Matrix Nitrite Modification Decreased CD46 Expression in ARPE-19

To model the aging effect on BM, we used a previously established approach of nitrite-modifying RPE-ECM. Immunohistochemistry and Western blot analysis showed that CD46 expression was greatest in ARPE-19 cells seeded onto untreated ECM (Fig. 2). This was significantly greater than that of ARPE-19 cells seeded onto nitrite-modified RPE-ECM (P = 0.003; Figs. 2A, 2B, 2E). ARPE-19 cells seeded onto nitrite-modified ECM, which was subsequently cleaned with Triton X-100 (nitrite-modified, cleaned treatment), expressed similar levels of CD46 as nitrite modified only, as measured by immunofluorescence (Figs. 2B, 2C). Western blot revealed slightly greater production of CD46 in ARPE-19 cells seeded onto nitrite-modified ECM that was subsequently cleaned with Triton X-100 than in cells on RPE-ECM that had been nitrite modified only, but this difference was not significant (P = 0.83; Fig. 2E). Coating previously nitrite-modified and cleaned ECM with extracellular proteins (nitrite-modified, cleaned, coated treatment) increased expression of CD46 in seeded ARPE-19 cells, such that levels were not significantly different from those of untreated ECM seeded ARPE-19 cells (fold change was −0.22, P = 0.32; Figs. 2A, 2D, 2E). Therefore, nitrite-modifying the surface of the ECM led to decreased levels of CD46 in ARPE-19 cells seeded onto this surface, relative to untreated ECM. This finding was reversed in ARPE-
19 cells by cleaning nitrite from the ECM and coating with extracellular proteins.

**siRNA Knockdown of CD46 in ARPE-19 Cells Leads to Increased VEGF Release**

To investigate whether CD46 may play a functional role in VEGF activation, we knocked down CD46 gene expression via siRNA. CD46 expression was significantly decreased after treatment with CD46 siRNA (Fig. 3A). Following knockdown of CD46, VEGF release was significantly increased on the apical surface of ARPE-19 cells, relative to random siRNA, by 32% ($P = 0.012$; Fig. 3B, left panel). Similarly, VEGF release was significantly increased on the basolateral surface of ARPE-19 cells, relative to random siRNA, by 19% ($P = 0.017$; Fig. 3B, right panel).

**The Effect of ECM Nitrite Modification on VEGF Activation in ARPE-19 Cells**

We investigated VEGF release in ARPE-19 cells seeded onto a similar system to that mentioned above. However, this time RPE-ECM was cultured onto Transwell permeable supports and basally exposed to 25% normal human serum. Vascular endothelial growth factor release was quantified in media from both the apical and basal surfaces of ARPE-19 cells by ELISA.

**Apical.** The apical release of VEGF was lowest in ARPE-19 cells seeded onto untreated ECM (Fig. 4A). Nitrite modification of the surface increased the release of VEGF apically by 286 pg/mL ($P < 0.001$; Fig. 4A). ARPE-19 cells seeded onto nitrite-modified ECM, which was subsequently cleaned with Triton X-100 (nitrite-modified, cleaned treatment), expressed less VEGF apically than cells on RPE-ECM that had been nitrite modified only, although this did not reach significance (−134 pg/mL ($P = 0.071$) and −142 pg/mL ($P = 0.068$), respectively, relative to nitrite modified only. Additionally, there was a moderate and significant difference between VEGF release in cells seeded onto cleaned ECM, coated with ECM proteins (nitrite-modified, cleaned, coated treatment), and that...
interestingly, there was a significant increase in VEGF activation from cells on nitrite-modified ECM that were nitrite modified, cleaned and coated, compared to those on ECM that were nitrite modified and cleaned only (increase was 22 pg/mL, \( P = 0.003 \); Fig. 4B). Additionally, there was a highly significant increase in VEGF activation from cells on ECM that were nitrite modified, cleaned, and coated, relative to those on untreated ECM (increase was 59 pg/mL, \( P < 0.001 \); Fig. 4B). Thus, nitrite-modifying the surface of ECM led to increased levels of VEGF in ARPE-19 cells relative to untreated ECM.

**Basal.** The basal release of VEGF was lowest in the media from ARPE-19 cells seeded onto untreated ECM (Fig. 4B). Nitrite modification of the surface increased basal release of VEGF by 58 pg/mL (\( P < 0.001 \); Fig. 4B). Cleaning the nitrite-modified surface with Triton X-100 (nitrite-modified, cleaned treatment) decreased VEGF relative to nitrite-modified ECM (\( P = 0.004 \); Fig. 4B). Interestingly, there was a significant increase between VEGF release by cells seeded onto cleaned ECM that were nitrite modified, cleaned and coated, compared with those on ECM that were nitrite modified and cleaned only (increase was 22 pg/mL, \( P = 0.003 \); Fig. 4B). Additionally, there was a highly significant increase in VEGF activation from cells on ECM that were nitrite modified, cleaned, and coated, relative to those on untreated ECM (increase was 59 pg/mL, \( P < 0.001 \); Fig. 4B). Thus, nitrite-modifying the surface of ECM led to increased levels of VEGF release by ARPE-19 cells seeded onto this surface, relative to those in untreated ECM. Subsequent cleaning of nitrite led to a trend toward restoring normal levels of VEGF. However, the addition of ECM proteins to previously nitrite-modified and cleaned ECM led to greater activation of VEGF levels than with those that were nitrite modified and cleaned only. Additionally, the overall pattern of responses across all ECM treatments was similar for both the apical and basal sides of the RPE.

**DISCUSSION**

CD46 is a complement regulatory protein that plays a major role in regulating the inflammatory response. While the association between CD46 and RPE atrophy still remains to be fully elucidated, this protein appears to play a critical role in RPE cell homeostasis on human BM. A decrease in CD46 expression is observed in the RPE of patients with GA, and this decrease is progressive. Thus, CD46 may be a critical component of the healthy RPE phenotype, and loss of CD46 may play a role in disease pathogenesis.

Herein, we investigated the expression of CD46 on nitrite-modified ECM derived from RPE cells, an in vitro model of "aging." We have previously demonstrated that nitrite modification of RPE-ECM has a significant effect on altering RPE attachment, proliferation, and survival. Additionally, we have previously demonstrated that nitrite modification of RPE-ECM has a detrimental effect on important RPE functions such as phagocytosis.

CD46 is expressed on the basolateral surface of polarized epithelium and is a critical component of cellular adhesion to ECM. In the current study, we demonstrated that there is polarized localization of CD46 in ARPE-19 cells when these cells are cultured onto a RPE-derived ECM. The basolateral polarization of CD46 supports the notion that this molecule protects the cell from complement-mediated damage, and its location suggests a role in maintaining a healthy RPE phenotype by contributing to cell adhesion to the basement membrane.

Treatment with anti-CD46 antibody (blocking antibody) leads to a dose-dependent inhibition of cell attachment to the basal lamina of human BM. It has been observed that alterations in RPE-CD46 expression progressively increase as RPE morphology deteriorates in the transition from healthy to a GA phenotype. Changes in CD46 expression are even seen during the early course of this disease, at a time when RPE cells are near normal morphology. This suggests that the protein’s association with RPE cells may form the functional units underlying normal adhesion mechanisms, maintaining a healthy RPE phenotype that is not proliferative or migratory.

Indeed, recently it has been shown that CD46-/- mice are more susceptible to experimental CNV owing to insufficient complement inhibition and increased VEGF. Moreover, it has been shown that expression of human CD46 by adenovirus infection reduces complement-mediated damage on murine ocular flat-mounts after challenge with human serum, suggesting that the protein may play a protective role in the retina during disease pathogenesis such as AMD.

In the current study, we demonstrated that culturing RPE cells on nitrite-modified RPE-ECM significantly reduced CD46 expression when compared to untreated RPE-ECM. This reduction in CD46 was reversed on nitrite-modified ECM that is subsequently cleaned and/or coated with extracellular proteins. It has been demonstrated that CD46, along with β1-integrin, plays an important role in RPE cell adhesion to basement membrane, and both bear a close association to one another. Aging and/or disease progression of BM reduces the ability of both proteins to adhere to ECM. Loss of these proteins may lead to the progression of AMD and make RPE cells more susceptible to complement exposure and eventual damage, as seen in both wet and dry AMD.

The direct role of CD46 expression on VEGF release was confirmed by siRNA treatment of ARPE-19 cells. Reduced
expression of CD46 using targeted siRNA resulted in increased VEGF release by RPE cells, suggesting a regulatory role for CD46 in VEGF release by RPE cells. Nitrite modification of ECM reduced CD46 expression and increased apical and basal VEGF release by ARPE-19 cells. To our knowledge, this is the first direct demonstration that age-related modification of the basement membrane leads to a change in both CD46 expression and VEGF release. It is difficult to not overemphasize the importance of the observation that changes of the basement membrane due to aging will decrease CD46 expression, because CD46 is an important inhibitor of the complement cascade; and reduced expression of CD46, which is located on the basolateral cell surface, will expose the RPE to activated complement components present in serum. This exposure could potentially exacerbate age-related disease.46,47 Our data are consistent with prior studies showing that complement activation from oxidative stress, induced by exposing RPE cells to H2O2 and normal human serum, will increase VEGF secretion.48 Our data are also consistent with the observation that CD46 knockout mice demonstrate an increased VEGF release upon the addition of ECM proteins. Alternatively, progressive loss of CD46 may contribute to atrophy as seen in advanced AMD or geographic atrophy.

The surprising observations that cleaning with the addition of ECM protein did not reduce VEGF release basally may be due to differing effects of individual ECM proteins on the behavior of epithelial and endothelial cells. For example, laminin increases VEGF release and promotes differentiation of endothelial cells.50 Studies conducted on RPE cell cultures have shown that the addition of soluble matrix proteins results in moderate increases in VEGF expression in an experimental model of hypoxia.51 Moreover, it has been shown that culturing of endothelial cells on different matrices such as vitronectin can increase expression of VEGF receptor.52 These observations may explain the increase in basolateral VEGF production in the eye and develop more severe disease in a laser-induced model of CNV.28

The relationship between aging in BM, CD46 expression, atrophy, and release of VEGF from the RPE is summarized in Figure 5. In the normal human eye, CD46 is expressed on the basolateral surface of the RPE and is closely associated with the β1-integrin subunit involved in cell adhesion. Vascular endothelial growth factor is released apically and basally by the RPE (Fig. 5A). Collagen cross-linking occurs in human eyes as a function of age, or can be induced in tissue culture by nitrite modification of the basement membrane. This reduces the expression of CD46 on the basement membrane of the RPE, which makes the cell more susceptible to complement-mediated damage (Fig. 5B). These age-related changes in basement membrane also increase the amount of VEGF that is released into the extracellular milieu, with an increase in both apical and basal VEGF release (Fig. 5C). In addition, the basement membrane changes seen early in the course of AMD will also lead to changes in VEGF that can enhance either CNV (basal VEGF release) or induce retinal angioma formation (apical VEGF release).53 Alternatively, progressive loss of CD46 may contribute to atrophy as seen in advanced GA (Fig. 5D).

Figure 5. Effects of nitrite exposure on CD46 and VEGF on RPE cells. Young (A) and aged (B) RPE cells are diagrammatically represented here. CD46 was expressed on the basolateral surface of the RPE and played a role in RPE cell adhesion to the basement membrane. Cross-linking of collagen in Bruch's membrane (through exposure to nitrite) reduced the expression of CD46 on the RPE. This reduction led to increased release of VEGF (C). CD46 also contributed to successful RPE cell adhesion to the basement membrane, and progressive loss of CD46 may contribute to atrophy (D). Thus, it is expected that loss of CD46 in aged or diseased Bruch's membrane increased VEGF production in the RPE and potentially contributed to the pathology seen in exudative AMD. Loss of CD46 alternatively contributed to the loss of RPE, resulting in the pathology seen in advanced AMD or geographic atrophy.

Numerous changes develop within human BM as a function of age, including the accumulation of drusen between the RPE and basement membrane, basal laminar and basal linear deposits,5,5 collagen cross-linking in the inner and outer collagen layer, calcification and fragmentation of the elastin layer,6 and membrane lipidization.6-8 Given the complexity of
these anatomic changes, it is important that in vitro models of BM pathology be developed to investigate the connection between basement membrane changes and disease pathogenesis. The use of nitrite-modified surfaces to mimic the effects of inflammation and aging serves as a relevant model to study the progression of diseases such as AMD. Nitrite exposure, as seen during chronic inflammation from aging and cigarette smoking, can directly modify ECM proteins and have a damaging effect on ECM structure. Thus, this exposure can lead to the structural damage associated with the cross-linking of proteins in the BM, one of the hallmark changes associated with basement membrane aging and disease. Nitrite modification of the ECM has been used previously as a model of basement membrane aging in AMD.

In conclusion, this study demonstrated that nitrite modification of RPE-derived basement membrane reduced CD46 expression in RPE cultured onto this surface. This leads to a downregulation of CD46, which places the cell at higher risk for apical and basal release of VEGF from the RPE. This can lead to neovascularization manifested as CNV or retinal angiomatous proliferation. Our results suggest a direct connection between the basement membrane changes seen in AMD and the cellular changes (atrophy and neovascularization) that are seen later in disease.

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