Peroxynitrite Upregulates Angiogenic Factors VEGF-A, BFGF, and HIF-1α in Human Corneal Limbal Epithelial Cells

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Purpose. Corneal neovascularization (NV) is a sight-threatening condition often associated with infection, inflammation, prolonged contact lens use, corneal burns, and acute corneal graft rejection. Macrophages recruited to the cornea release nitric oxide (NO) and superoxide anion (O_2^-), which react together to form the highly toxic molecule peroxynitrite ($ONOO^-$). The role of $ONOO^-$ in upregulating multiple angiogenic factors in cultured human corneal limbal epithelial (HCLE) cells was investigated.

Methods. Human corneal limbal epithelial cells were incubated with 500 μM of $ONOO^-$ donor for various times. VEGF-A, BFGF, and hypoxic-inducible factor-alpha (HIF-1α) were investigated via Western blot and RT-PCR was performed for VEGF. Functional assays using human umbilical vein endothelial cells (HUVEC) used conditioned media from $ONOO^-$-exposed HCLE cells. Secreted VEGF from conditioned media was detected and analyzed using ELISA.

Results. Increased angiogenic factors were observed as early as 4 hours after HCLE exposure to $ONOO^-$. HIF-1 expression was seen at 4, 6, and 8 hours post-$ONOO^-$ exposure ($P < 0.05$). BFGF expression was elevated at 4 hours and peaked at 8 hours after treatment with $ONOO^-$ ($P < 0.005$). Increased VEGF-A gene expression was observed at 6 and 8 hours post-$ONOO^-$ treatment. Functional assays using conditioned media showed increased HUVEC migration and tube formation.

Conclusions. Exposure to elevated extracellular concentrations of $ONOO^-$ results in upregulation of angiogenic factors in HCLE cells. It is possible that, in the setting of inflammation or infection, that exposure to $ONOO^-$ could be one contributor to the complex initiators of corneal NV. Validation in vivo would identify an additional potential control point for corneal NV.

Keywords: corneal epithelium, neovascularization, Oxi, epithelial cells

Corneal neovascularization (NV) results from the pathological ingrowth of blood vessels from the limbal vascular plexus into the cornea. Corneal NV is a sight-threatening condition that contributes significantly to eye disease and vision loss. Approximately 4% of patients presenting for general ophthalmologic care in the United States have been reported to have a diagnosis of corneal NV, suggesting that 1.4 million Americans per year are seen annually for this condition.[1] Worldwide, corneal NV caused by infectious diseases have devastating consequences that affect 400 million individuals per year.[1]

Pathological NV of the cornea has been associated with a disequilibrium between pro- and antiangiogenic factors.[2] VEGF-A and BFGF are endogenously present at low levels within the cornea.[3] Following corneal injuries or infections, recruitment of inflammatory cells leads to an upregulation of proangiogenic factors.[4] Increased levels of VEGF and BFGF may arise indirectly from infiltrating inflammatory cells.[5–9] However, it is also possible that increased proangiogenic factors may originate in the corneal epithelium.

In addition to proinflammatory cytokines, infiltrating macrophages, monocytes, and leukocytes release reactive oxygen and nitrogen species.[10–12] Nitric oxide (NO) and superoxide anion (O_2^-) react together to form the highly toxic molecule peroxynitrite ($ONOO^-$).[13] Elevated concentrations of $ONOO^-$ have been reported in many inflammatory diseases,[14] including spinal cord injury, septic shock, diabetic retinopathy and microangiopathy, atherosclerosis, and colon adenomas.[15–20] The primary role of $ONOO^-$ in ocular inflammation is poorly understood; however, in animal models of endotoxin-induced uveitis and experimental autoimmune uveitis, elevated levels of NO and $ONOO^-$ have been well documented.[21–23]

Cytotoxic and proangiogenic properties and actions of $ONOO^-$ have been characterized in several cell types, including smooth muscle cells and vascular endothelial cells.[24] Microvascular endothelial cells exposed to exogenous $ONOO^-$ increase

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VEGF mRNA and VEGF protein expression in a time- and dose-dependent manner.[24]

The potential role for ONOO⁻ to upregulate expression of angiogenic factors by corneal epithelium has not been previously reported. This study demonstrated that elevated extracellular concentration of ONOO⁻ produces an upregulation of the angiogenic factors VEGF, hypoxic-inducible factor-alpha (HIF-1α), and BFGF in a human corneal limbal epithelial (HCLE) cell line. This observation suggests that pathologic NV of the cornea after inflammation could possibly result in part from stimulation of local expression of proangiogenic factors by the corneal epithelium.

Methods

Cell Culture

Human corneal limbal epithelial cells (gift of Ilene Gibson, Schepens Eye Research Institute, Harvard Medical School) are a spontaneously arising human corneal limbal epithelial cell line. Human corneal limbal epithelial cells were grown and incubated with serum-free keratinocyte media with human recombinant epidermal growth factor 1-53 and bovine pituitary extract (KSFM; Life Technologies, Grand Island, NY) at 37°C, 5% CO_2. Human corneal limbal epithelial cells were seeded onto six-well culture plates and incubated for 2 days until cells reached confluence.

ONOO⁻ Treatments

SIN-1 (3-morpholinosyndnomine) has been used as an ONOO⁻ donor in previous studies.[13–15,21,23,24] It decomposes spontaneously in neutral aqueous media, consuming oxygen to release equimolar amounts of NO and O_2^- simultaneously.[13] These two agents react rapidly to produce ONOO⁻.[13] The concentrations of ONOO⁻ used in this study are less than what has previously been used in other pathological models of ocular disease. In one study,[21] uveitis was produced in rabbits by injection of 5 mM SIN-1, in another study,[23] 20 mM SIN-1 induced endothelial cell damage and corneal edema. The half-life of ONOO⁻ at pH 7.4 is only a few seconds.[13] To mimic the endogenous effects of ONOO⁻, exposure time and exogenous concentrations of ONOO⁻ must be taken into account. Under physiological conditions, endogenous ONOO⁻ is continuously produced at low concentrations. Thus, higher concentrations and extended exposure times with exogenous ONOO⁻ are necessary to mimic the biological responses seen with endogenous ONOO⁻.[24]

The SIN-1 (≥98% purity; Sigma-Aldrich, St. Louis, MO) was added to keratinocyte serum-free medium (KSFM) of cultured HCLE cells at concentrations ranging from 0.125 to 1 mM. Based on the reported erythrocyte sedimentation rate of SIN-1 degradation and rate of ONOO⁻ production,[25] the concentration of SIN-1 used in the present study would yield 3.75 to 30 μM ONOO⁻ total production. ONOO⁻ itself has a half-life of less than 1 second in aqueous solution.[26] Under these conditions, the donor is relatively stable, having a half-life (t_{50}) of 14 to 26 minutes.[27,28] The SIN-1 was dissolved in KSFM and incubated with HCLE cells after 30 minutes. Maximum ONOO⁻ production occurs when SIN-1 is left in solution for at least 30 minutes.[29] Because the reaction of NO and O_2^- occurs virtually instantly, it maximizes the effects of ONOO⁻ and limits the opportunity for NO and O_2^- to exert independent effects.[29]

Human corneal limbal epithelial cells cultured with KSFM were used to study the effects of each tested concentration of SIN-1. The duration of incubation with SIN-1 ranged from 2 to 24 hours (three replications per time point). After the incubation with SIN-1 was complete, HCLE cells were washed with PBS, lysed, and boiled at 95°C for 5 minutes with Laemmli buffer for Western blot, and the conditioned media collected for functional assay studies.

Control Conditions

Three control conditions were used. First, 2.0 mM of uric acid (UA), a natural and highly specific scavenger of ONOO⁻,[30] was introduced into 1 mM SIN-1 and KSFM 30 minutes before incubation with HCLE cells. Second, a replacement KSFM was introduced in lieu of SIN-1. Third, 1 mM SIN-1 was added to KSFM 24 hours before incubation with HCLE cells. This enabled evaluation of the effects of the ONOO⁻-depleted donor since the time course of dissociation of ONOO⁻ approximates the time course of degradation of the donor (t_{50} ~14–26 minutes).

Tube Formation and Migration Assays

Human umbilical vein endothelial cells (HUVECs; ATTC, Manassas, VA) were used in assays to determine if the secreted VEGF was active in inducing physiologic responses by endothelial cells.

For tube formation assays, the HUVEC cells were cultured in VEC media (VEC Technologies, Inc., Rensselaer, NY). Sterile cover slips were placed in 24-well plates; 50 μL Geltrex (Invitrogen, Carlsbad, CA) per cm^2 was added to each cover slip surface and incubated for 30 minutes at 37°C to allow the gel to solidify. Human umbilical vein endothelial cells were harvested and seeded onto glass cover slips coated with Geltrex. Human corneal limbal epithelial cell–conditioned media was added to each well and incubated for 4 hours, after which 2 μL Calcein (Life Technologies) was added to the HUVEC culture media and incubated for an additional 30 minutes. Conditioned media was carefully aspirated, the cover slips were mounted onto a slide, and evaluated and quantified using fluorescein microscopy by a researcher who was masked with respect to the sample identity.

For the Migration Assays

Human umbilical vein endothelial cells were seeded into the upper compartment of the Transwell insert of Boyden chambers (24-well plate; Corning, Tewksbury, MA). Human corneal limbal epithelial cell–conditioned media was added to the lower compartment of each Boyden chamber well. After 4 hours of incubation with the conditioned media, the Transwell inserts were fixed with 4% paraformaldehyde and stained with 0.2% crystal violet for 5 minutes. The inserts were carefully washed with distilled water and dried overnight. The inserts were imaged and HUVEC migration was quantified via light microscopy.

ELISA

Conditioned media from HCLE cells treated with ONOO⁻ were collected at previously stated time points. Secreted VEGF from conditioned media was detected and analyzed using ELISA kit (R&D Systems, Minneapolis, MN), following the manufacturer's instructions. Fifty microliters of assay diluent and 200 μL of conditioned media was added to each well of the precoated 96-well plate. The plate was allowed to incubate for 2 hours at room temperature, and washed with manufacturer's wash buffer three times. Two hundred microliters of anti-VEGF conjugated antibody was added to each well for 2 hours at room temperature. After 2 hours, plates were washed three times.

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Substrate solution was added to each well, and incubated for 20 minutes at room temperature in the dark. Last, stop solution was added to each well and gently tapped to ensure thorough mixing. Optical density was determined using a Ultramark 550 microplate reader (Bio-Rad, Hercules, CA) set to 450 nm.

Western Blot Analysis

Western blot analysis was performed as previously described.[31] Briefly, cell protein was isolated using radio immunoprecipitation assay buffer containing protease and phosphatase inhibitors (Roche, Indianapolis, IN), and the protein concentration determined via Bradford protein assay (Bio-Rad). A total of 20 μg protein was loaded in each lane of an SDS-PAGE gradient gel in reducing conditions. Proteins were transferred to nitrocellulose membranes (GE Healthcare, Buckinghamshire, UK) for 1 hour at 100 V. The membrane was then blocked with 5% nonfat milk in 0.1% TBS-Tween solution. Blots were incubated for 1 hour with primary antibody at a dilution of 1:500 for HIF-1α (BD Biosciences, San Jose, CA), 1:200 for BFGF (R&D Systems), and 1:5000 for β-actin (US Biological, Salem, MA). Horseradish peroxidase–conjugated goat anti-rabbit (BD Bioscience) or horseradish peroxidase–conjugated goat anti-mouse (Southern Biotech, Birmingham, AL) was exposed to the blots at a 1:2000 dilution. The blots were then developed using a chemiluminescence assay to visualize bound antibody (Millipore, Billerica, MA). The Western blot analyses were quantified with Image J software (National Institutes of Health, Bethesda, MD). The blots were digitized with a flatbed scanner, and the band density was measured by using Image J. To account for loading variability, β-actin was used to normalize each sample. At least three independent experiments were performed and the results were evaluated for statistical significance with a Student's *t*-test (unpaired, two-tail). A level of *P* less than 0.05 was considered to be statistically significant.

VEGF RT-PCR

RNA was isolated with the RNeasy kit (Qiagen, Chatsworth, CA) according to the manufacturer's instructions. After initial recovery, DNA was depleted by treatment with DNase I (Qiagen); 3 μg of total RNA, isolated as described above, was used as starting material, to which 1 μL random primers was added, as well as RNase-free water to total volume of 8 μL. The RNA mix was heated at 65°C for 5 minutes and then chilled on ice. First-strand reaction mix and Superscript III/RNaseOUT Enzyme mix (Invitrogen) were added to the RNA mix, and incubated at 25°C for 10 minutes, followed by 50°C for 50 minutes. The reaction was inactivated at 85°C for 5 minutes, followed by the addition of 1 μL (2 U/μL) of RNase H and incubation at 37°C for 20 minutes.

The PCR conditions were as follows: initial activation step at 95°C for 10 minutes, 29 cycles each of melting at 95°C for 15 seconds, annealing at 60°C for 1 minute, and extension at 72°C for 1 minute. A negative control without template was included in each replicate to assess the overall specificity of the reaction. The PCR products were analyzed on a 2% agarose gel to confirm the size of the amplified product. Band density was normalized to glyceraldehyde 3-phosphate dehydrogenase (GAPDH) and quantitated using NIH Image J software. The experiments were repeated in quadruplicate and the SEM is reported.

The primer sequences were as follows: VEGFxxx forward 5′-AGCTACTGCCATCCAATCGC-3′, reverse 5′-GGGCGAATCC AATTCCAAGAG-3′. In parallel, GAPDH forward 5′-AGTACTCC GTGTGGATCGGC-3′ and reverse 5′-GCTGATCCACATCTGC TGGA-3′ primers were used as an endogenous control for normalization.

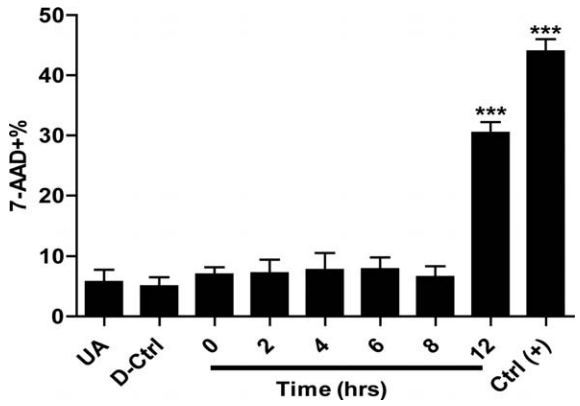

FIGURE 1. ONOO⁻ does not cause early cell death. HCLE cells treated with 500 μM SIN-1 for 0 to 12 hours, and the percentage of dead cells was detected by flow cytometry after 7-AAD staining. Three independent negative controls included UA (2 mM UA + 1 mM SIN-1); D-ctrl (depleted SIN-1 donor and KSFM media added in lieu of SIN-1); and 0 hour (no treatment). The positive control (Ctrl [+]) was HCLE cells heat-treated at 56°C for 5 minutes, and then incubated on ice for an additional 5 minutes. Cell death was significant ($P < 0.005$) after 12 hours of treatment with ONOO⁻.

Flow Cytometry Analysis of Cell Death

Human corneal limbal epithelial cells were seeded in six-well plates at a density of 1×10^6 cells/well. Once the cells reached 80% to 90% confluence, they were incubated with 500 μM SIN-1 for 0, 2, 4, 6, 8, and 12 hours. Samples were stained with 7-amino actinomycin (7-AAD; BD Biosciences), 5 μL (0.25 μg) per million cells, in fluorescence-activated cell sorter (FACS) buffer (PBS with 0.5% bovine serum albumin), at room temperature. The 7-AAD staining was used to identify and quantitate dead cells by flow cytometry. All samples were immediately acquired on a FACSCalibur machine (BD Biosciences) with CellQuest Pro software (BD Biosciences). Data were analyzed using FlowJo software (TreeStar, Ashland, OR).

RESULTS

Cell Integrity Following 500 μM of SIN-1 Exposure

To determine whether 500 μM SIN-1 had any cytotoxic effects on HCLE cells, a 7-AAD cell viability assay was performed. When HCLE cells were incubated with 500 μM SIN-1 for 2, 4, 6, and 8 hours, there was an increase in cell death of 0.5% to 2.0% compared with the different negative controls. Three negative control conditions were used. First, 2.0 mM UA was introduced into 1 mM SIN-1 + KSFM 30 minutes before incubation with HCLE cells for an additional 8 hours. Second, KSFM without SIN-1 was used at 0 hour, and HCLE cells were incubated in KSFM alone for 8 hours. Third, 1 mM SIN-1 was added to KSFM media 24 hours before incubation with HCLE cells (D-Ctrl) to create an ONOO⁻ depletion. Significant cell death ($P < 0.004$) was observed when HCLE cells were incubated with ONOO⁻ for 12 hours. Four independent trials were completed (Fig. 1).

HIF-1α Expression Increases Following Exposure of HCLE Cells to ONOO⁻

Hypoxic-inducible factor-alpha is a well-known regulator of VEGF-A and BFGF. Protein levels of HIF-1α were measured by Western blot, after treatment of HCLE cells with 500 μM SIN-1. Band density was quantified with normalization to the β-actin loading control. Following incubation with ONOO⁻, HIF-1α

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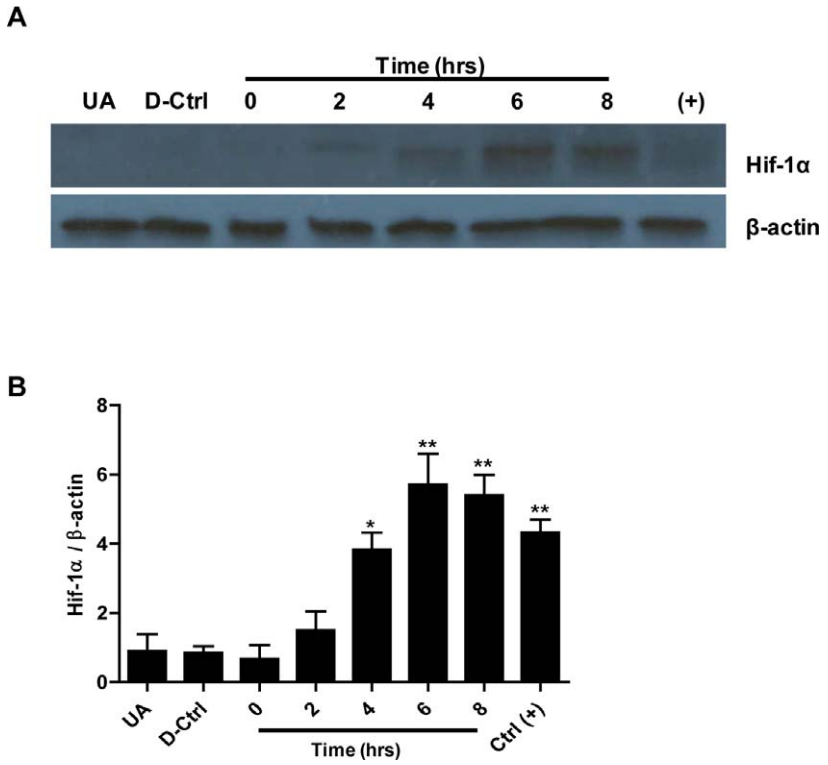

FIGURE 2. HIF-1α expression increases after exposure to ONOO⁻. Protein levels of HIF-1α were measured by Western blot after treatment of HCLE cells with 500 μM SIN-1. (**A**) Representative immunoblots. (**B**) Band density, normalized to β-actin loading control. Increased HIF-1α protein expression was significantly elevated at 4, 6, and 8 hours of incubation with ONOO⁻ ($P < 0.05$). A 5-fold increase in HIF-1α protein expression was seen at 8 hours of incubation with ONOO⁻, compared with negative controls. Data are compiled from four independent experiments.

protein expression was significantly elevated at 4, 6, and 8 hours of incubation with ONOO⁻ ($P < 0.005$; Fig. 2). At 8 hours, the increase was 5-fold compared with negative controls. Data are compiled from four independent experiments (Fig. 2). Notably, there was no increase in signal transducer and activator of transcription 3 (STAT3) activation by the HCLE cells after exposure to ONOO⁻ (data not shown).

ONOO⁻ Increases VEGF-A mRNA Expression

To examine whether ONOO⁻ exposure alters VEGF-A mRNA expression, total VEGF-A mRNA expression was measured via RT-PCR. Figure 3A is representative of four independent experiments. When HCLE cells were exposed to 500 μM SIN-1, there was a detectable increase in VEGF-A mRNA starting at 4 hours (8.0- to 8.5-fold increase), and was statistically significant at 6 hours ($P < 0.0014$) and 8 hours ($P < 0.0083$; Fig. 3B). Three independent negative control conditions validated that the increase in VEGF-A mRNA was due to ONOO⁻ exposure. When replacement KSFM media was introduced in lieu of ONOO⁻ (0 hour), or when HCLE cells were incubated with a depleted ONOO⁻ donor (D-Ctrl), and finally when HCLE cells were coincubated with 2.0 mM UA and 1 mM SIN-1 (UA), VEGF-A mRNA levels were undetectable. Thus, the increase in VEGF-A mRNA levels was due to ONOO⁻ exposure.

Mean VEGF Expression Increases After Exposure to 500 μM SIN-1

To determine whether the increase in VEGF-A mRNA expression was concordant with VEGF-A protein expression, human VEGF assays were completed using conditioned media of HCLE cells exposed to 500 μM of SIN-1. Significant increase ($P < 0.0001$) in VEGF expression was observed at 6 and 8 hours post-ONOO⁻ treatment, with approximately 18- to 20-fold increase in VEGF expression over negative control (Fig. 3C).

BFGF Protein Expression Increases After Exposure to ONOO⁻

Another endogenous angiogenic factor found within the cornea epithelium is BFGF, so its protein levels were measured by Western blot analysis in HCLE cells after incubation with 500 μM SIN-1. Cell extracts were fractionated by 4% to 20% SDS-PAGE gradient gel, and band density, normalized to β-actin loading control, was quantified. Following incubation with ONOO⁻ for 6 and 8 hours, there was a 9.6- to 13.4-fold increase in BFGF compared with negative controls, respectively ($P < 0.0001$). Data were compiled from four independent experiments (Fig. 4).

Functional Significance of ONOO⁻-Induced Angiogenic Factor Expression by HCLE

ONOO's⁻ ability to upregulate expression of proangiogenic factors by HCLE cells is a previously unreported finding. To further validate the functional significance of ONOO⁻-mediated angiogenic factor expression, capillary tube formation and migration were assessed using the HUVEC cells as a read-out for the presence of proangiogenic factors.

The number of tubes formed from HUVEC cells were examined following incubation with conditioned media from HCLE cells exposed to 500 μM SIN-1. Greater numbers of tubes were formed when HUVEC cells were incubated with conditioned media from HCLE cells exposed to 4, 6, and 8

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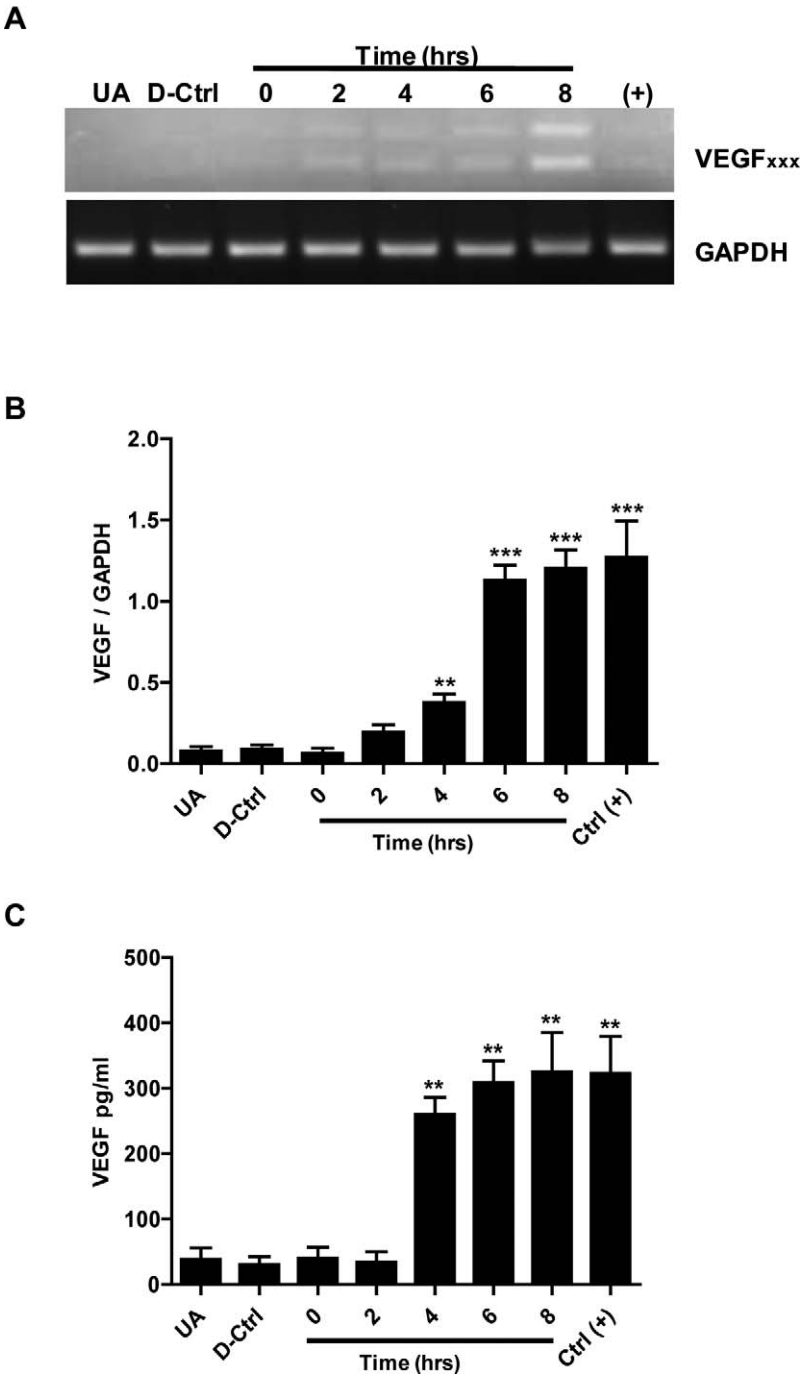

FIGURE 3. ONOO⁻ increases VEGF-A mRNA expression. HCLE cells were exposed to 500 μM SIN-1 for 0 to 8 hours, and evaluated for (**A**, **B**) VEGF-A mRNA and (**C**) VEGF protein detected by ELISA of HCLE medium after period of SIN-1 exposure. Three independent negative control conditions (UA, D-Ctrl, 0 hour) were used (see Fig. 1). Data from four independent experiments were tabulated. (**A**) Representative images of VEGF-A mRNA. (**B**) Mean VEGF-A mRNA expression: 500 μM SIN-1 resulted in a 1.5-fold increase in VEGF-A mRNA levels compared with negative controls at 6 and 8 hours of exposure with ONOO⁻ ($P < 0.0005$). (**C**) VEGF protein levels in culture medium showed a 3-fold increase that was significant ($P < 0.05$) at 4, 6, and 8 hours.

hours of ONOO⁻. Images are representative of four independent experiments (Fig. 5A).

The number of tube vessels formed from HUVEC cells grown in conditioned media from HCLE cells treated with 500 μM SIN-1or with negative controls were counted and averaged from at least six random fields. There was a significant increase ($P < 0.05$) in the number of tube formation after exposure to conditioned media from cells that had been exposed for 4 hours to ONOO⁻. Specifically, following 6 and 8 hours of incubation with ONOO⁻, there was a 5.4- and 5.1-fold increase in the number of tubes formed compared with negative controls, respectively. Data were compiled from four independent experiments (Fig. 5B).

Human umbilical vein epithelial cell migration through a Transwell filter after incubation with conditioned media from HCLE cells exposed to 500 μM SIN-1 was assessed. Figure 6A is

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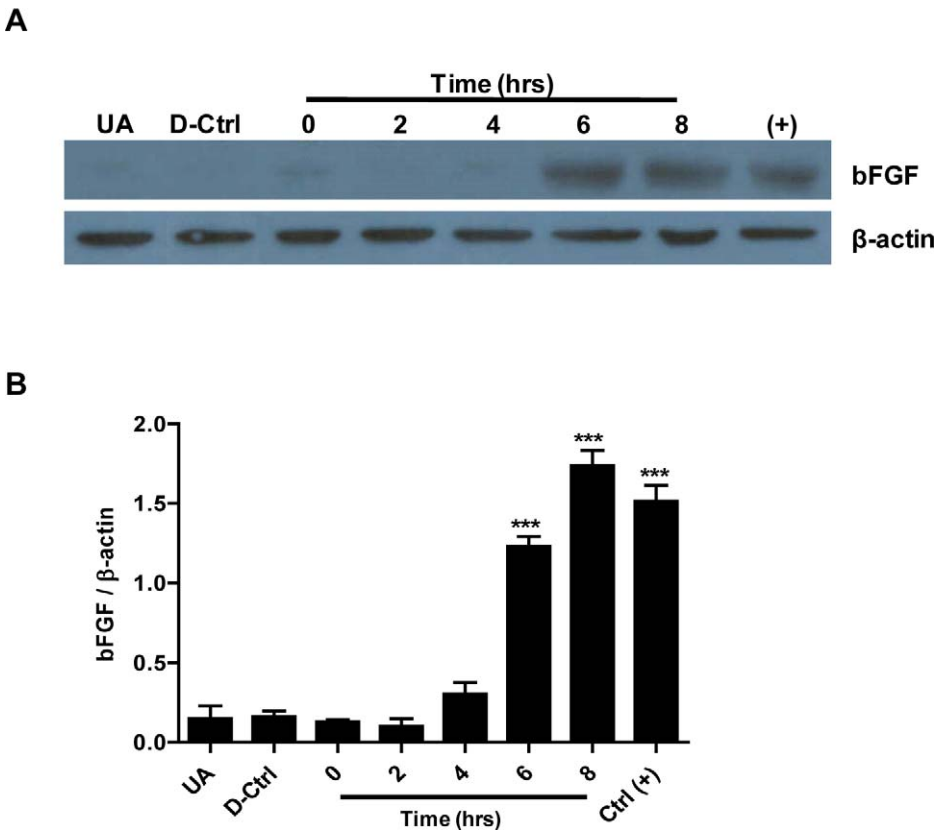

FIGURE 4. BFGF protein expression increases after exposure to ONOO⁻. HCLE cells cultured with SIN-1 as described in Figure 3 were assayed for BFGF protein levels by Western blot analysis. (**A**) Representative immunoblots. (**B**) Band density, normalized to β-actin loading control, was quantified. Following incubation with ONOO⁻ for 6 and 8 hours, there was a 1.2- and 1.8-fold increase in BFGF compared with negative controls (UA, D-Ctrl, and 0 hour), respectively ($P < 0.0005$). Data were compiled from four independent experiments.

representative of HUVEC cell migration after incubation with conditioned media from HCLE cells exposed to 500 μM SIN-1 for 0 to 8 hours. Conditioned media derived from HCLE cells that were exposed to ONOO⁻ resulted in a 5.5- and 6.6-fold increase in HUVEC migration and 6 and 8 hours ($P < 0.05$), respectively. In contrast, when conditioned media was prepared from HCLE cells exposed to one of the control conditions, no increase in HUVEC migration was observed. Data were compiled from four independent experiments (Fig. 6B). This indicates that ONOO⁻ indirectly promotes HUVEC migration, possibly through VEGF-, HIF-1α–, and BFGF-mediated pathways.

DISCUSSION

Corneal NV is a sight-threating condition that is associated with the pathological growth of blood vessels. Angiogenesis is common in tumor growth and corneal NV.[32,33] In tumors, VEGF-A has been shown to be the most potent angiogenic factor,[34] and known to promote tumor growth.[34,35] In the eye, VEGF-A expression has been shown to be upregulated after corneal injury and infection in both human and animal models.[5,36] Similarly, VEGF expression also has been correlated with pathological blood vessel growth in vivo, after corneal injury.[37,38] In addition to VEGF, BFGF and HIF-1α are also endogenous angiogenic factors within the corneal epithelium.[32,33] However, there is an incomplete understanding of the cellular mechanisms relating local ocular inflammation to angiogenic factor induction, specifically whether the epithelium itself can contribute to the local production of proangiogenic factors.

Elevated reactive nitrogen and oxygen species have been documented within the cornea after infectious or inflammatory processes. Specifically, it has been reported that NO and O_2^-, released by infiltrating macrophages, react to produce the highly toxic molecule ONOO⁻.[24] The purpose of the present study was to test the hypothesis that ONOO⁻ directly upregulates angiogenic factors, VEGF-A, BFGF, and HIF-1α, in HCLE cells. The results support this hypothesis by revealing an increased expression in VEGF-A mRNA, and upregulation in BFGF and HIF-1α protein expression, after incubation of HCLE cells with (0.125–1 mM) of the ONOO⁻ donor SIN-1. We also demonstrated that conditioned media of HCLE cells treated with ONOO⁻ resulted in increased cell migration and tube formation, further validating the finding that ONOO⁻ upregulates angiogenic factors and that these factors are biologically active. The absence of VEGF-A mRNA, BFGF, and HIF-1α protein expression when SIN-1 was coadministered with UA, a specific scavenger of ONOO⁻, supports the notion that the upregulation of angiogenic factors was attributable to ONOO⁻ per se rather than NO or O_2^-, as UA does not bind NO.[30]

BFGF is an endogenous angiogenic factor found within the corneal epithelium.[39] BFGF exerts enhanced proliferative activity on endothelial cells during hypoxia. It is reported that this is due to low-affinity heparin sulfate BFGF binding sites.[40,41] Human endothelial cells subjected to hypoxic conditions upregulate BFGF, in a process mediated via HIF-1α.[42] To our knowledge, the present study is the first report that treating HCLE cells with ONOO⁻ results in an upregulation of BFGF protein expression. To our knowledge, this study is

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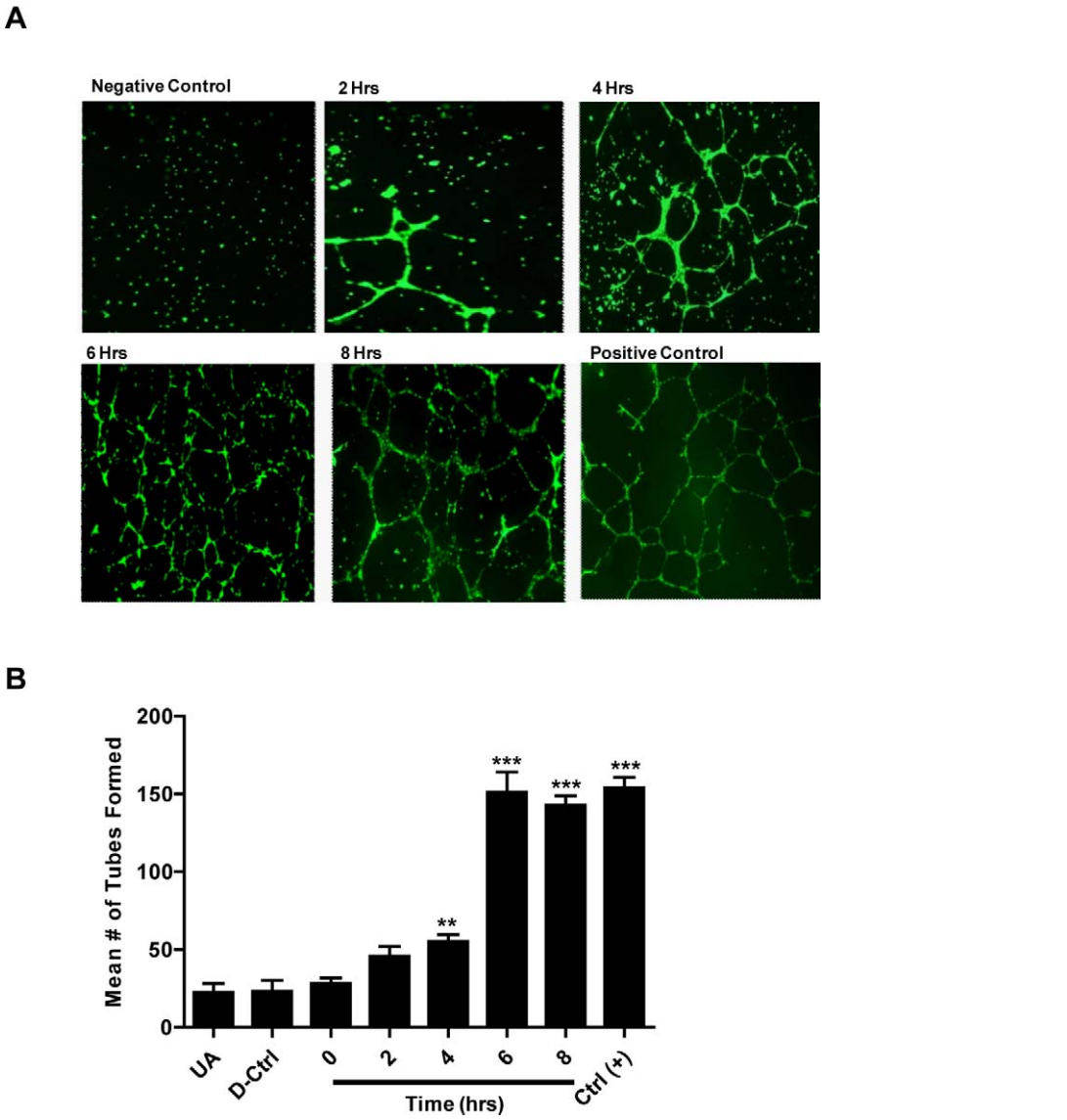

FIGURE 5. Induction of HUVEC tube formation by HCLE cell–conditioned medium. HCLE cells were exposed to 500 μM SIN-1 for indicated times, and conditioned medium was collected and tested for induction of HUVEC tube formation. (**A**) Representative images of tube formation. (**B**) Quantitation of results tabulated from four independent experiments. Elevated tube formation was induced with conditioned media treated with ONOO⁻ for more than 2 hours compared with negative controls (D-Ctrl and 0 hour; see Fig. 1); the increase was significant ($P < 0.0005$) for later conditioned media (4, 6, 8 hours) reaching 3-fold elevation.

the first to report an increase in VEGF, BFGF, and HIF-1α expression in HCLE cells following treatment with ONOO⁻.

We further tested the hypothesis that ONOO⁻ upregulation of HIF-1α expression is a molecular mechanism by which ONOO⁻ increases VEGF and BFGF expression in HCLE cells. In mammalian cells, the response to oxygen deprivation is mediated by the transcription factor, HIF-1. Hypoxic-inducible factor-1 has two subunits: HIF-1β, which is constitutively expressed, and HIF-1α, which is strongly upregulated under hypoxic conditions.[42,43] The transcriptional response of HIF-1α to hypoxia is poorly understood in corneal limbal epithelium cells. In vitro models of various breast cancer cell lines that have been exposed to hypoxic conditions have identified HIF-1 as the "master regulator of the transcriptional response to oxygen deprivation."[42,43] It has been well documented that hypoxic tissues express pathological concentration of angiogenic factors, such as VEGF and BFGF, and that this is mediated through the actions of HIF-1α.[40,44] We show in this study that upregulation of VEGF mRNA, and of BFGF protein, is strongly correlated with increased HIF-1α protein expression. This suggests that HIF-1α may mediate angiogenic factors in HCLE cells following their treatment with ONOO⁻. In fact, we observed that the increase in HIF-1α precedes both the increase in VEGF and BFGF, suggesting that HIF-1α was operating as the upstream regulator of these angiogenic factors. However, increase in STAT3 activation, but not HIF-1α was observed when microvascular endothelial cells were treated with 100 μM ONOO⁻.[24] This observation may indicate that ONOO⁻ works differently in endothelial cells as compared with epithelial cells, or that the concentration of ONOO⁻ was not sufficient to increase HIF-1α expression in endothelial cells.

Regulation of HIF-1α is complex and may involve both increased protein expression and decreased degradation.[45] Signaling through activation of SRC or HER2 kinases, or alternatively through epidermal growth factor receptor or

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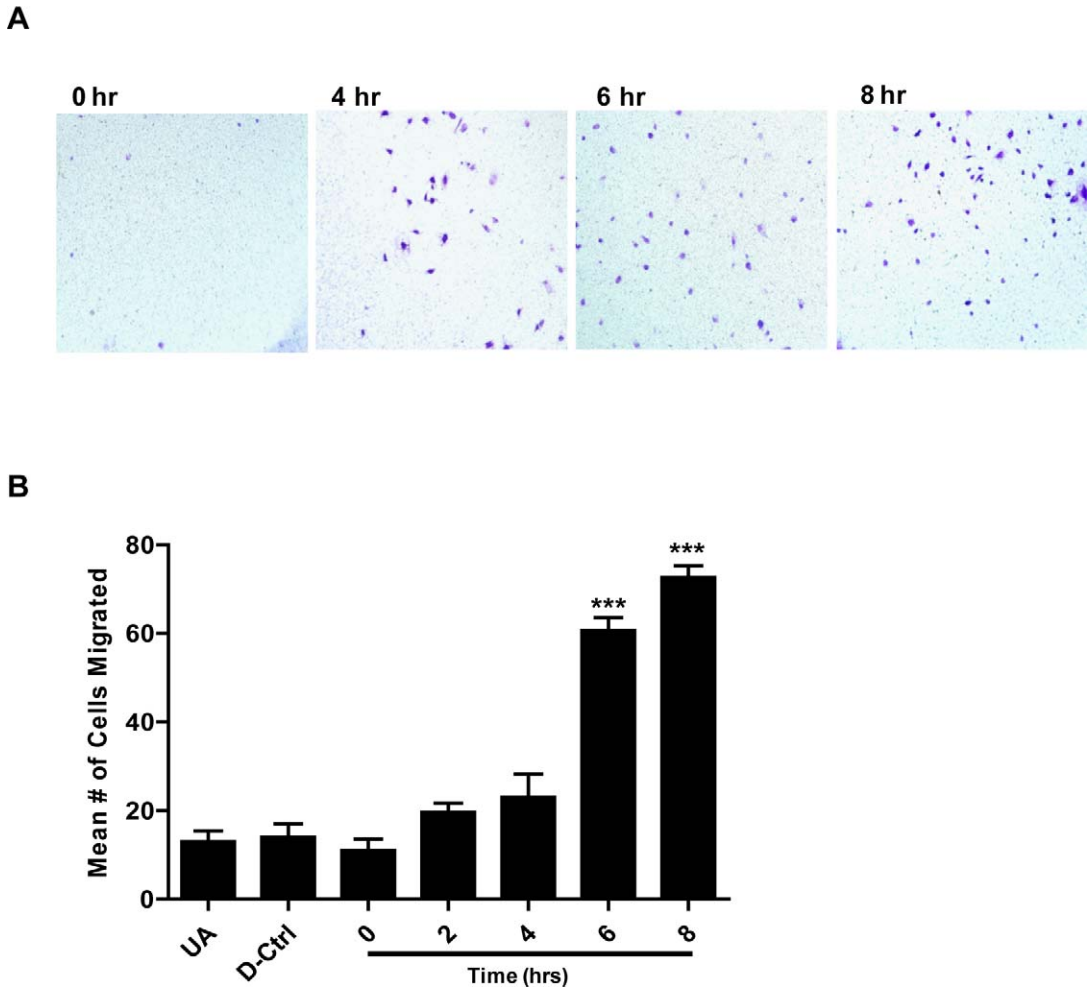

FIGURE 6. Induction of HUVEC migration by HCLE cell–conditioned medium. HUVEC cells were cultured on 24-Transwell plates in normal HUVEC media. Conditioned media from HCLE cells exposed to 500 μM SIN-1 was added to the bottom of the Transwell plate. HUVEC cells were incubated for 6 hours, and then stained with crystal violet, and migrated cells were quantified. (**A**) Representative images of Transwell migration. (**B**) Quantitation of results from four independent experiments. Conditioned media (6 and 8 hours) induced a 4- and 5-fold increase in HUVEC migration ($P < 0.0005$) compared with negative controls (D-Ctrl).

insulin-like growth factor receptor, leads to activation of the phosphatidylinositol 3-kinase (PI3K)/AKT pathway, leading to increased translation of HIF-1α. Alternatively, a decrease in ubiquitination or protein degradation can also lead to increases in HIF-1α. Regulation of HIF-1α by NO, O_2^-, and TNF-α has been previously studied, increases in HIF-1α by these inflammatory mediators required the activation of the PI3K/AKT signaling pathway.[46,47] Because NO and O_2^- react to form $ONOO^-$, it is highly likely that $ONOO^-$ activates HIF-1α through a similar signaling pathway. However, additional studies would be required to fully characterize the signaling that results in increased HIF-1α and VEGF expression in the HCLE cells.

Although a single variable or factor cannot explain the complex mechanisms involved in corneal NV, one possible stimulus may involve the recruitment of macrophages to the cornea during infection or inflammation. Infiltration of macrophages, monocytes, and leukocytes is known to release various proinflammatory cytokines, and reactive oxygen and nitrogen species. In addition to a direct effect by the infiltrating cells and their products, the present study suggests that stimulation of proangiogenic factor expression by the corneal epithelium in response to local reactive oxygen species may potentially play an additional role in pathologic NV. Additional studies looking at the mechanisms of regulation of expression of proangiogenic agents by corneal epithelium in response to $ONOO^-$, as well as the specific response of the microvascular endothelium of the limbus, would be necessary to further characterize this hypothesis.

Our study is the first to show that $ONOO^-$ directly stimulates VEGF, BFGF, and HIF-1α expression in HCLE cells. Induced local expression of proangiogenic factors by the corneal epithelium, if validated in vivo, is a potential pathway for future therapeutic interventions in the treatment and control of corneal NV.

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