

Hypoxia-Inducible Factor-Dependent Expression of Angiopoietin-Like 4 by Conjunctival Epithelial Cells Promotes the Angiogenic Phenotype of Pterygia

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PURPOSE. Disappointing results from clinical studies assessing the efficacy of therapies targeting vascular endothelial growth factor (VEGF) for the treatment of pterygia suggest that other angiogenic mediators may also play a role in its development. We therefore explore the relative contribution of VEGF, hypoxia-inducible factor (HIF)-1 α (the transcription factor that regulates VEGF expression in ocular neovascular disease), and a second HIF-regulated mediator, angiopoietin-like 4 (ANGPTL4), to the angiogenic phenotype of pterygia.

METHODS. Expression of HIF-1 α , VEGF and ANGPTL4 were examined in surgically excised pterygia, and in immortalized human (ih) and primary rabbit (pr) conjunctival epithelial cells (CjECs). Endothelial cell (EC) tubule formation assays using media conditioned by ihCjECs in the presence or absence of inducers/inhibitors of HIF-1 or RNA interference (RNAi) targeting VEGF, ANGPTL4, or both were used to assess their relative contribution to the angiogenic potential of these cells.

RESULTS. HIF-1 α and VEGF expression were detected in 6/6 surgically excised pterygia and localized to CjECs. Accumulation of HIF-1 α in was confirmed in ihCjECs and prCjECs, including stratified prCjECs grown on collagen vitrigel, and resulted in expression of VEGF and the promotion of EC tubule formation; the latter effect was partially blocked using RNAi targeting VEGF mRNA expression. We demonstrate expression of a second HIF-regulated angiogenic mediator, ANGPTL4, in CjECs in culture and in surgically excised pterygia. RNAi targeting ANGPTL4 inhibited EC tubule formation and was additive to RNAi targeting VEGF.

CONCLUSIONS. Our results support the development of therapies targeting both ANGPTL4 and VEGF for the treatment of patients with pterygia.

Keywords: pterygia, angiogenesis, vascular endothelial growth factor, angiopoietin-like 4, hypoxia-inducible factor

A pterygium is a nonneoplastic, degenerative, fibrovascular proliferation of conjunctival tissue that extends onto the cornea that can affect up to 22% of the population in certain geographic regions.¹ Inflammation of pterygia, resulting in

conjunctival hyperemia and irritation, is not uncommon. Advanced pterygia can lead to significant visual impairment if they encroach on the central visual axis, cause the formation of a corneal dellen, or promote irregular astigmatism. Current

treatment options for symptomatic pterygia are limited, and surgical excision remains the most effective intervention. Despite numerous modifications to the surgical approach, recurrences remain common.²

While the precise molecular etiology of pterygia has not been determined, the secretion of angiogenic growth factors that promote fibrovascular proliferation is believed to play an important causative role.^{3,4} Considerable effort has focused on elucidating the role of one potent angiogenic stimulator, vascular endothelial growth factor (VEGF), in the formation of pterygia.⁵⁻⁸ VEGF has been implicated in the pathogenesis of a growing list of ocular neovascular diseases in which its expression is dependent on the transcription factor, hypoxia-inducible factor (HIF)-1.^{9,10} In these diseases, accumulation of the α subunit (HIF-1 α) of the heterodimeric HIF-1 results in increased expression of VEGF, which in turn promotes the development of pathological angiogenesis. While the relationship between HIF-1 and VEGF in retinal neovascular disease has been an active focus of research, the relative contribution of HIF-1 and HIF-regulated angiogenic mediators (in addition to VEGF) to the angiogenic phenotype of pterygia has only recently come under investigation^{11,12} and remains poorly understood.

The observation that VEGF expression is increased in excised pterygia has prompted speculation that VEGF inhibition may be a rational antiangiogenic approach as a primary or adjuvant treatment for pterygia patients.¹³⁻¹⁵ Enthusiasm for antiangiogenic therapies for pterygia has been dampened, however, by disappointing results from clinical studies evaluating topical, intrascleral, or subconjunctival anti-VEGF therapies to treat—or prevent recurrence of—pterygia.¹⁶⁻²⁴ One explanation may be that other angiogenic factors, in addition to VEGF, may contribute to the vascular component of pterygia. Here, we examine the relative contribution of HIF-1, VEGF, and a second recently identified HIF-regulated angiogenic mediator, angiopoietin-like 4 (ANGPTL4),²⁵⁻²⁷ to the angiogenic phenotype of pterygia.

MATERIALS AND METHODS

Reagents

VEGF and ANGPTL4 (DuoSet) ELISA kits were purchased from R&D Systems (Minneapolis, MN, USA). Predesigned control (scrambled), HIF-1 α , ANGPTL4, and VEGF small-interfering RNAs (siRNAs) were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Lipofectamine RNAiMAX transfection reagent was obtained from Life Technologies (Carlsbad, CA, USA). Digoxin and desferrioxamine (DFO) were obtained from Sigma-Aldrich Corp. (St. Louis, MO, USA). Dimethylxalylglycine (DMOG) was obtained from Cayman Chemicals (Ann Arbor, MI, USA).

Cell Culture

Primary rabbit (pr) conjunctival epithelial cells (CjECs) were obtained from enucleated New Zealand white rabbit eyes with eyelids attached (Pel-Freez Biologicals, Rogers, AR, USA) as previously described.²⁸ Briefly, isolated rabbit conjunctiva tissue was carefully dissected from rabbit eyes and placed in 12-well culture plate in Dulbecco's modified Eagle medium (DMEM): Nutrient Mixture F-12 (DMEM/F-12; Corning, Manassas, VA, USA) with Dispase II (1 U/mL, Roche Diagnostics, Basel, Switzerland) at 4°C for overnight. The CjECs were then carefully scraped from the conjunctiva and cultured in Accutase cell detachment solution (Sigma-Aldrich Corp.) at 37°C for 20 minutes. After neutralizing with DMEM/F-12, the

cells were filtered and counted. PrCjECs were passaged in bronchial epithelial cell growth medium (BEGM; Lonza, Walkersville, MD, USA). Immortalized human (ih) CjEC line was kindly provided by Ilene K. Gipson, PhD (Schepens Eye Research Institute, Harvard Medical School) and cultured with keratinocyte-serum-free medium (K-SFM, Gibco, Grand Island, NY, USA) supplemented with human recombinant epidermal growth factor (EGF; 5 ng/mL), bovine pituitary extract (50 μ g/mL), and 1% penicillin/streptomycin (Corning) as previously described.²⁹ Cells were cultured at 37°C in a 5% CO₂ incubator. For hypoxia experiments, cells were exposed to 1% O₂ using an Oxygen Controller Glove Box (Coy Laboratory Products, Inc., Grass Lake, MI, USA), equilibrated with a gas mixture containing 1% O₂, 5% CO₂, and 94% N₂ at 37°C.³⁰

Vitrigel Culture

The collagen vitrigel (CV) membrane was prepared as previously described.^{31,32} Briefly, type I collagen solution from bovine dermis (5 mg/mL, Cosmo Bio, Tokyo, Japan) was mixed at equal volume with DMEM (Thermo Fisher Scientific, Waltham, MA, USA) on ice, which contains 10% fetal bovine serum (FBS; Thermo Fisher Scientific) and 20 nM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid buffer (HEPES; Thermo Fisher Scientific). The mixture was then transferred to Transwell inserts (polyester membrane with 0.4- μ m pore size, 300 μ L/cm²; Corning) and incubated at 37°C for 2 hours. After gelation, the collagen gel was vitrified under 40% relative humidity (RH) at 40°C for 1 week. Before cell seeding, the gel membrane was rinsed with phosphate-buffered saline (PBS; Thermo Fisher Scientific) three times.

PrCjEC were then seeded on Transwell containing CV membranes at a density of 2×10^4 cells/cm² and cultured at 37°C with 5% CO₂. BEGM was added to both upper and lower compartments until the cells reached confluence after about 5 days. Afterwards, medium was switched to a 1:1 mixture of BEGM and DMEM/F12 to induce stratification as previously described.²⁸ Induction medium was added to only the lower compartment for airlifting culture, allowing the cells to be exposed to the air-liquid interface. Cells were kept in air-lifting culture for 1 week before hypoxia exposure.

For histology, prCjECs cultured on CV membranes were fixed with 4% paraformaldehyde (PFA; Electron Microscopy Sciences, Hatfield, PA, USA) and washed with PBS. The CV/cell samples were further dehydrated through ethanol gradients, cleared in xylenes, and embedded in paraffin. Five-micrometer thick sections were used for hematoxylin and eosin (H&E; Sigma-Aldrich Corp.) staining, according to the manufacturer's manuals. Images of stained samples were taken on the Axio Imager 2 fluorescent microscope (Carl Zeiss, Oberkochen, Germany).

siRNA Transfection

Cells were seeded and grown to 60% to 80% confluence prior to transfection. Lipofectamine RNAiMAX reagent was diluted in Opti-MEM medium. Thirty picomoles of siRNA from stock of 10 μ M was diluted in Opti-MEM medium. Diluted siRNA was added to diluted Lipofectamine RNAiMAX reagent (1:1 ratio) and incubated for 5 minutes at room temperature. siRNA-lipid complex was added to cells and incubated at 37°C for 24 hours. The medium was then washed out and cells were ready for experiments.

Western Blot Assays

Cells in culture dishes were washed with PBS and lysed using radioimmunoprecipitation assay (RIPA) buffer (Sigma-Aldrich

Corp.) with 10% protease inhibitor cocktail (Cell Signaling, Danvers, MA, USA). Cell lysates were then solubilized in lithium dodecyl sulfate (LDS)-sample buffer (Life Technologies) and incubated for 5 minutes at 95°C. Lysates were subjected to 4% to 15% gradient SDS/PAGE (Life Technologies). After blocking the membrane with 5% milk (Bio-Rad, Hercules, CA, USA), the membrane was then incubated with mouse anti-human HIF-1 α (610959; BD, Franklin Lakes, NJ, USA) or mouse anti-rabbit HIF-1 α (Thermo Fisher Scientific, PA1-16601) or with mouse anti-human GAPDH monoclonal antibody (Fitzgerald, Acton, MA, USA) or mouse anti-rabbit GAPDH monoclonal antibody (NB300-328SS; Novusbio, Littleton, CO, USA) overnight at 4°C. After washing, the membrane was incubated with (HRP)-conjugated anti-mouse or anti-rabbit IgG (Cell Signaling) for 1 hour and then visualized with ECL Super Signal West Femto (Thermo Scientific, Rockford, IL, USA). Western blot scans are representative of at least three independent experiments.

Quantitative Real-Time RT-PCR

mRNA was isolated from cultured cells with PureLink RNA Mini Kit (Life Technologies), and cDNA was prepared with MuLV Reverse Transcriptase (Applied Biosystems, Carlsbad, CA, USA). Quantitative real-time PCR was performed with Power SYBR Green PCR Master Mix (Applied Biosystems) and StepOnePlus Real-Time PCR Detection System (Life Technologies). β -actin and β 2-microglobulin were used for normalization of human cell lines or for rabbit cells, respectively. Primers of human cell lines for quantitative PCR (qPCR) include: VEGF, forward- GGGCAGAATCATCAGAAAGT and reverse- TGGTGATGTTGGACTCCTCA; ANGPTL4, forward-GGACACGGCCTATAGCCTG and reverse-CTCTTGCGCGCATTCCTTGTC; β -actin, forward-CTCTCCAGCCTTCCTTCCT and reverse-AGCACTGTGTTGGCGTACAG. Primers of rabbit cells for qPCR include: VEGF, forward-GCTCTACCTCCACATGCCA and reverse-TCACCACTTCGTGGGGTTTATT; ANGPTL4, forward-GAGCCAGGTTGGTCTCCTGA and reverse-GCCATTTGGGGAGCCTCTT; β 2-microglobulin, forward-CAGTTCACCCGCCTCAC and reverse-CACATGTCTCGATCCCAG.

Enzyme-Linked Immunosorbent Assay

Conditioned medium diluted 1:1 were analyzed for ANGPTL4 and VEGF with ELISAs performed according to the manufacturer's protocols (R&D Systems).

Endothelial Cell (EC) Tubule Formation Assay

EC tubule formation assay was performed using growth factor-reduced Matrigel (Corning; 356231). Fifty microliters of Matrigel was added into a prechilled 96-well plate and placed in a 37°C CO₂ incubator for 30 minutes. Human dermal microvascular endothelial cells (HMVECs) were then counted and plated at 2×10^4 cells/well on the Matrigel in a 96-well plate. Eighteen hours later, images were captured and analyzed using ImageJ software to measure total tube length.³³ Tubule formation assay with conditioned medium from ihCjECs was performed with an addition of 100 μ L/well of conditioned medium to the cell suspension prior to adding into the Matrigel-coated wells.

Immunohistochemistry

An ABC system (Dako, Glostrup, Denmark) was performed in paraffin-embedded human tissue as previously described.^{25,27,34} Primary antibodies used include: CD34 (Cova-

nance, Princeton, NJ, USA), HIF-1 α (ABCAM, Cambridge, MA, USA), ANGPTL-4 (ABCAM), and VEGF (Santa Cruz Biotechnology) after dilution in Tris-buffered saline (TBS) with 1% bovine serum albumen (BSA). All immunohistochemical reagents, including antibodies, were identical for all specimens.

Statistical Analysis

Results from cell culture models are shown as mean \pm SEM from at least three independent experiments. Statistical differences between groups were determined by Student's *t*-test or 1-way ANOVA as indicated. Statistical analysis was performed using Microsoft Office and Prism 6.0 software (GraphPad). **P* < 0.05; ***P* < 0.01; ****P* < 0.001; and *****P* < 0.0001.

RESULTS

HIF-1 α and VEGF Are Expressed in Surgically Excised Pterygia and Localize to the Conjunctival Epithelium

While the molecular pathology of pterygia is not well understood, the prominent fibrovascular component appears to play an important role in its growth. Immunohistochemical examination of the "apex" of surgically excised pterygia, which resides over the cornea, demonstrated prominent vasculature (highlighted by CD34-positive vascular ECs) overlying the cornea (Fig. 1A). Expression of the potent angiogenic mediator, VEGF, was most evident in the overlying epithelium in 6/6 pterygia examined (Fig. 1A). Similarly, expression of the transcription factor, HIF-1 α , the master regulator of angiogenic mediators in ocular neovascular disease, was prominent in the conjunctival epithelium (Fig. 1A). Similar results were observed in the "body" of the pterygia, which resides over the conjunctiva, in which expression of both VEGF and HIF-1 α was most notable in the conjunctival epithelium (Fig. 1B). By contrast, expression of VEGF and HIF-1 α was not readily detected in normal conjunctival epithelium (Fig. 1C).

HIF-1 α Accumulation Is Necessary and Sufficient for the Angiogenic Phenotype of Hypoxic CjECs

We next set out to assess the contribution of HIF-1 α accumulation in CjECs to the angiogenic phenotype of pterygia. To this end, we subjected ihCjEC²⁹ to hypoxia (1% O₂ for 4 hours) and observed an accumulation of HIF-1 α (Fig. 2A). Treatment with digoxin, an inhibitor of HIF-1 α protein accumulation,^{35,36} inhibited this effect, while treatment with a pharmacologic HIF inducer, DFO or DMOG, resulted in accumulation of HIF-1 α in ihCjECs under nonhypoxic conditions (20% O₂; Fig. 2A). Similarly, exposure of primary CjECs isolated from rabbit eyes (prCjECs) to hypoxia or a HIF inducer (DMOG) resulted in HIF-1 α accumulation (Fig. 2B). Similar results were obtained in prCjECs grown on a collagen-based membrane, CV, on which CjECs grow as a multilayered (stratified) epithelium, similar to that observed in human conjunctiva (Figs. 2C, 2D).

We next took advantage of the CjEC culture system as a model to study the angiogenic response that drives the development of pterygia. To this end, we examined the ability of media conditioned by ihCjECs to promote the formation of tubules by immortalized human microvascular ECs (HMVECs). We observed a potent increase in tubule formation by HMVECs treated with media conditioned by ihCjECs exposed to hypoxia (Fig. 2E; Supplementary Fig. S1A). The increase in EC tubule formation was effectively inhibited using the HIF-1 α inhibitor

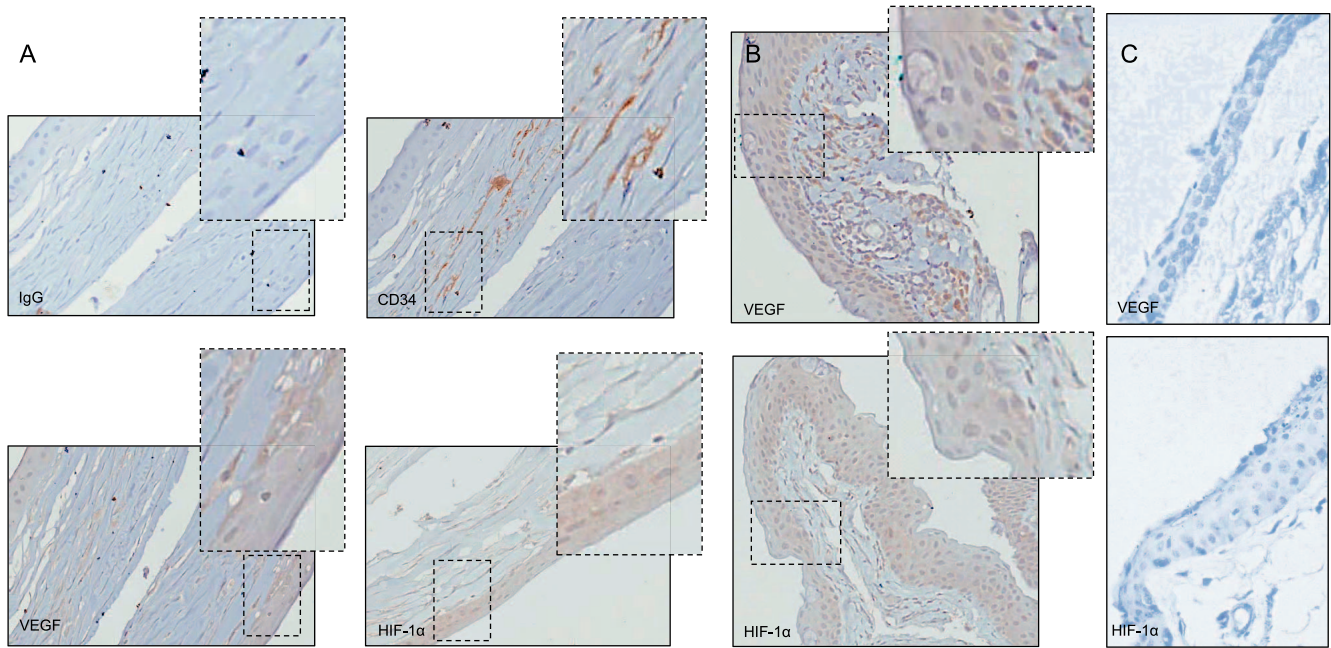


FIGURE 1. HIF-1 α expression is detected in conjunctival epithelium from surgically excised pterygia. (A) Immunohistochemical staining of the “apex” of a pterygium for CD34 highlighting vascular ECs in the fibrovascular stroma. VEGF and HIF-1 α expression is detected in the overlying epithelium. IgG was used as a negative control. (B) Immunohistochemical staining of the “body” of a pterygium similarly demonstrates expression of VEGF and HIF-1 α in the CjECs. Similar results were observed in 6/6 pterygia. (C) Immunohistochemical staining of VEGF and HIF-1 α in “normal” conjunctiva of autopsy eyes with no known history of anterior segment disease. Similar results were observed in 4/4 autopsy eyes.

digoxin (Fig. 2F; Supplementary Fig. S1B). Similarly, tubule formation was markedly increased in HMVECs treated with media conditioned by ihCjECs exposed to DFO or DMOG as compared to media conditioned by ihCjECs exposed to 20% O₂ (Fig. 2G; Supplementary Fig. S1C). The effect of DFO was inhibited by digoxin (Fig. 2H; Supplementary Fig. S1D).

VEGF Secretion Is Not Sufficient to Explain the Angiogenic Potential of Cultured CjECs

To determine whether expression of the potent HIF-regulated angiogenic factor VEGF is responsible for the increased HIF-dependent angiogenic phenotype of CjECs, we next examined

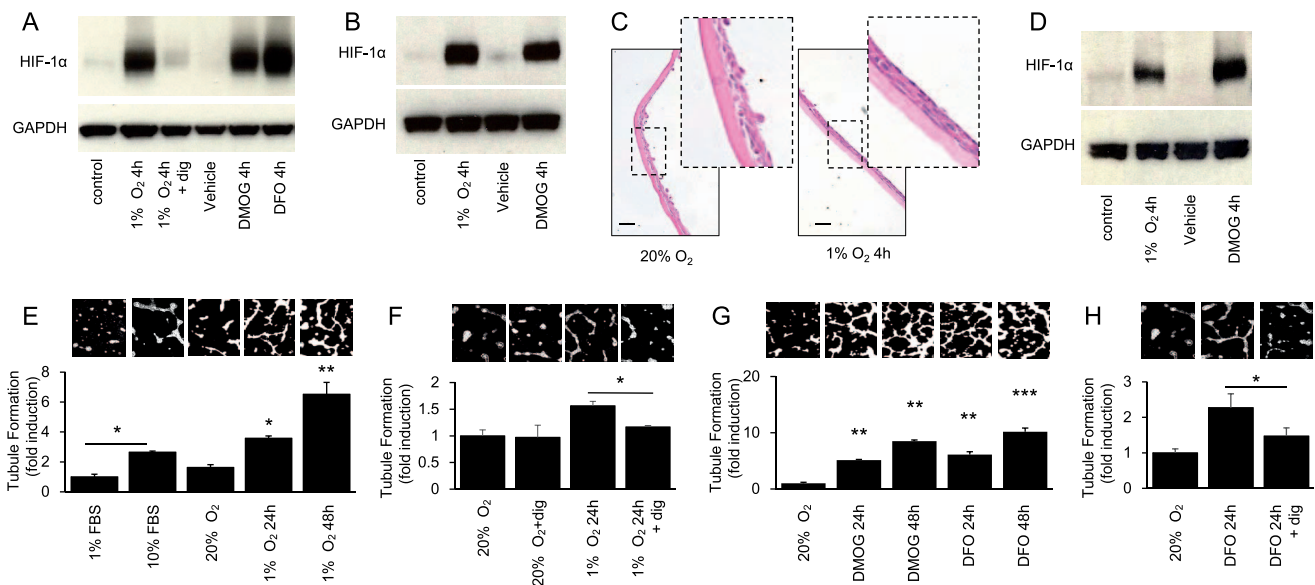


FIGURE 2. Accumulation of HIF-1 α in cultured CjECs results in the secretion of angiogenic mediators. (A) Immunoblot for HIF-1 α in ihCjECs exposed to hypoxia (1% O₂) or a HIF inducer (100 μ M DFO or 300 μ M DMOG), in normoxia (20% O₂) for 4 hours. One hundred nanomolar digoxin was used to inhibit HIF-1 α accumulation. (B) Immunoblot for HIF-1 α in prCjECs exposed to 1% O₂ or 300 μ M DMOG for 4 hours. (C) H&E stain of stratified prCjECs grown on vitrigel. (D) Immunoblot for HIF-1 α in stratified prCjECs grown on vitrigel exposed to 1% O₂ or 300 μ M DMOG for 4 hours. (E–H) EC tubule formation by HMVECs treated with conditioned media from ihCjECs exposed to 1% O₂ (E, F) or 100 μ M DFO or 300 μ M DMOG (G, H), in the absence (E, G) or presence (F, H) of 100 nM digoxin, compared to media conditioned by cells exposed to 20% O₂ for 24 hours. Ten percent FBS was used as a positive control.

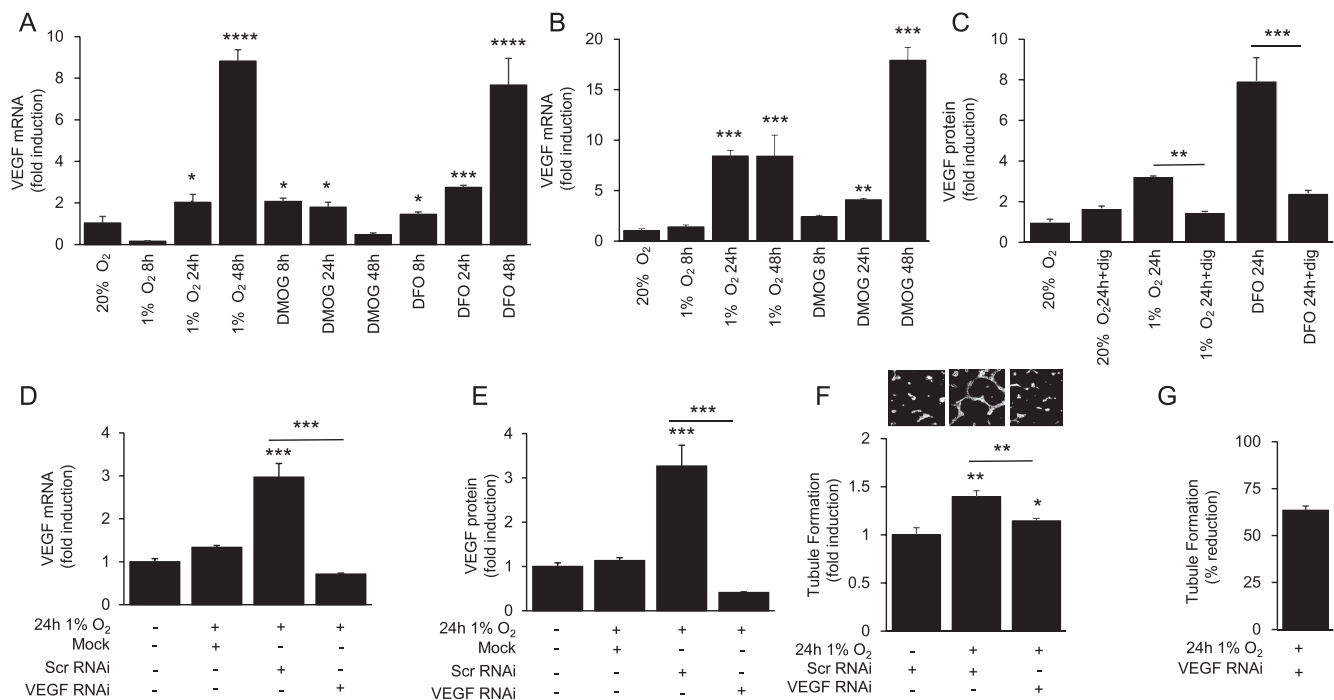


FIGURE 3. VEGF expression by cultured ihCjECs is not sufficient to explain the angiogenic potential of these cells. (A, B) VEGF mRNA expression in ihCjECs (A) or prCjECs (B) exposed to 1% O₂, 100 μ M DFO, or 300 μ M DMOG for 8, 24, or 48 hours. (C) VEGF protein secretion in ihCjECs exposed to 1% O₂, 100 μ M DFO, or 300 μ M DMOG for 24 hours. One hundred nanomolar digoxin was used to inhibit HIF-1 α accumulation. (D, E) VEGF mRNA expression (D) and protein secretion (E) compared by ihCjECs transfected with RNAi targeting VEGF and exposed to 1% O₂ for 24 hours. Mock transfected (Mock) or scrambled RNAi (Scr) transfected cells were used as a control. (F, G) EC tubule formation by HMVECs treated with conditioned media from ihCjECs transfected with VEGF RNAi and exposed to 1% O₂ for 24 hours reported as fold induction (F) or percent reduction (G).

the expression of VEGF by CjECs in response to HIF-1 α accumulation. Exposure of ihCjECs to hypoxia or HIF inducer (DMOG or DFO) resulted in an increase in VEGF mRNA expression (Fig. 3A). Similar results were observed in prCjECs (Fig. 3B). In turn, this resulted in a HIF-dependent increase in VEGF protein secretion by CjECs (Fig. 3C). However, while RNA interference (RNAi) targeting VEGF effectively inhibited VEGF mRNA expression (Fig. 3D) and protein secretion (Fig. 3E) by hypoxic ihCjECs, it only partially inhibited the angiogenic potential of hypoxic CjECs (Figs. 3F, 3G; Supplementary Fig. S2).

ANGPTL4 Expression Is Increased in Response to HIF-1 α Accumulation in Cultured CjECs

The observation that blocking VEGF expression only partially inhibits the angiogenic potential of CjECs is consistent with prior observations from clinical studies that antiangiogenic therapies targeting VEGF are not sufficient to prevent the growth or pterygia in treated patients.¹⁹ One explanation for these observations is that other angiogenic mediators may contribute to the prominent fibrovascular component of pterygia. We therefore set out to assess whether another HIF-regulated angiogenic factor contributed to the ability of CjECs to promote angiogenesis. To this end, we examined the expression in CjECs of ANGPTL4, which contributes to pathological angiogenesis in diabetic eye disease.^{25,27,34} We observed increased ANGPTL4 mRNA expression and protein secretion following treatment of ihCjECs with hypoxia, DMOG, or DFO (Figs. 4A, 4B). Pharmacologic inhibition of HIF-1 with digoxin effectively blocked ANGPTL4 mRNA expression and protein secretion (Figs. 4C, 4D). Similar results were observed in prCjECs (Fig. 4E).

ANGPTL4 Expression Is Detected in Surgically Excised Pterygia and Localizes to the Conjunctival Epithelium

We next examined the expression of ANGPTL4 in surgically excised pterygia. Immunohistochemical staining demonstrated expression of ANGPTL4 in the conjunctival epithelium and, to a lesser extent, the underlying fibrovascular stroma in 6/6 pterygia (Fig. 5A). The pattern of expression for ANGPTL4 was similar to that observed for VEGF. By contrast, expression of ANGPTL4 was not readily detected in normal conjunctival epithelium (Fig. 5B).

Inhibition of ANGPTL4 Expression by CjEC Reduces Their Angiogenic Potential

To evaluate the contribution of ANGPTL4 to the angiogenic potential of CjECs, we used RNAi to knock down expression of ANGPTL4 in ihCjECs exposed to hypoxia. RNAi targeting ANGPTL4 inhibited hypoxia-induced ANGPTL4 mRNA expression and protein secretion (Figs. 6A, 6B), but did not influence VEGF mRNA expression (Fig. 6C). Similar results were observed for DFO-induced promotion of ANGPTL4 mRNA and protein expression (Figs. 6D, 6E). Conditioned media from ihCjECs exposed to hypoxia or DFO and treated with RNAi resulted in a marked reduction in the promotion of EC tubule formation (Figs. 6F, 6G; Supplementary Fig. S3), similar to targeting VEGF (Fig. 3).

Inhibition of Both ANGPTL4 and VEGF Expression by CjEC Abolishes Their Angiogenic Potential

To evaluate the therapeutic potential of combined therapy targeting both ANGPTL4 and VEGF to inhibit the angiogenic

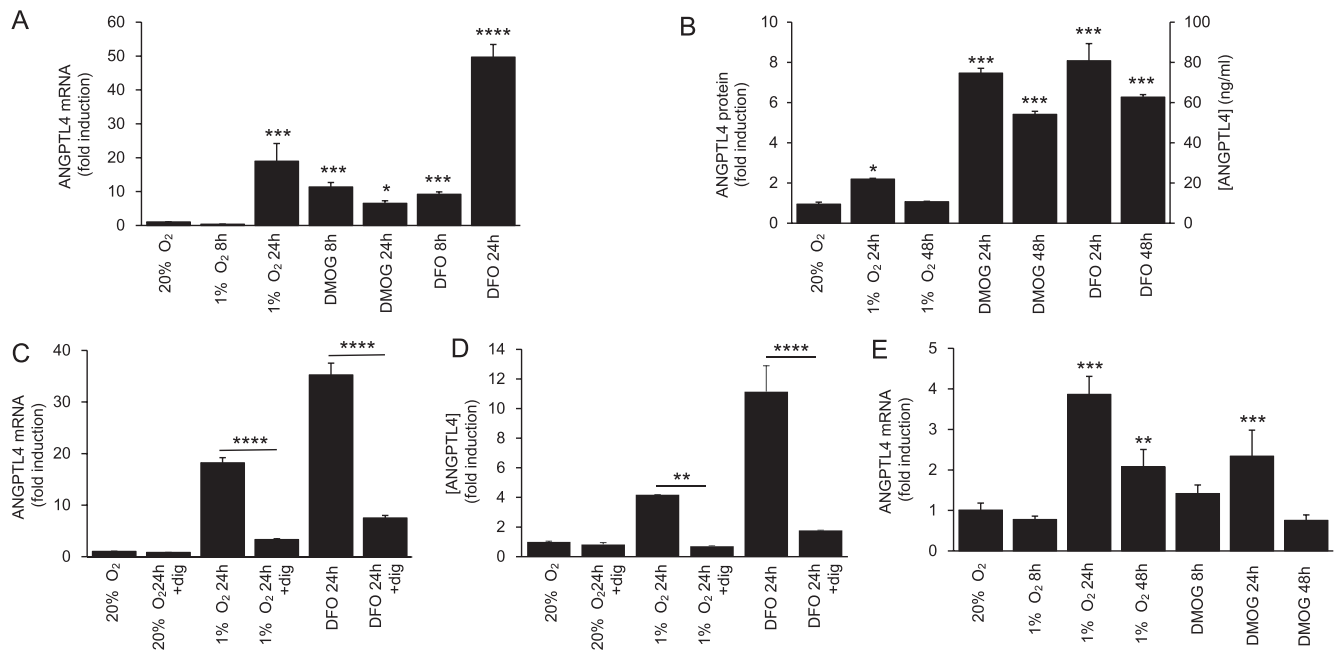


FIGURE 4. ANGPTL4 secretion from cultured CjECs is markedly increased in response to HIF-1 α accumulation. (A–D) ANGPTL4 mRNA expression (A, C) and protein secretion (B, D) by ihCjECs exposed to 1% O₂, 100 μ M DFO, or 300 μ M DMOG in the absence (A, B) or presence (C, D) of 100 nM digoxin. (E) ANGPTL4 mRNA expression by prCjECs exposed to 1% O₂ or 300 μ M DMOG for 8, 24, or 48 hours.

potential of CjECs, we used RNAi to knock down expression of ANGPTL4, VEGF, or both in ihCjECs exposed to hypoxia. RNAi targeting ANGPTL4 inhibited hypoxia-induced ANGPTL4 mRNA expression and protein secretion (Figs. 7A, 7B), but did not influence VEGF mRNA expression or protein secretion (Figs. 7C, 7D). Similarly, RNAi targeting VEGF inhibited hypoxia-induced VEGF mRNA expression and protein secretion (Figs. 7C, 7D), but did not influence ANGPTL4 mRNA expression or protein secretion (Figs. 7A, 7B). RNAi targeting both ANGPTL4 and VEGF inhibited mRNA expression and protein secretion for both (Figs. 7A–D). Conditioned media

from ihCjECs exposed to hypoxia and treated with RNAi targeting either ANGPTL4 or VEGF partially inhibited the promotion of EC tubule formation, while RNAi targeting both ANGPTL4 and VEGF completely abolished the ability of CjECs to promote EC tubule formation (Figs. 7E, 7F; Supplementary Fig. S4).

DISCUSSION

Pterygia are a common cause of cosmetic disfigurement within the equatorial zone and can lead to significant vision loss when

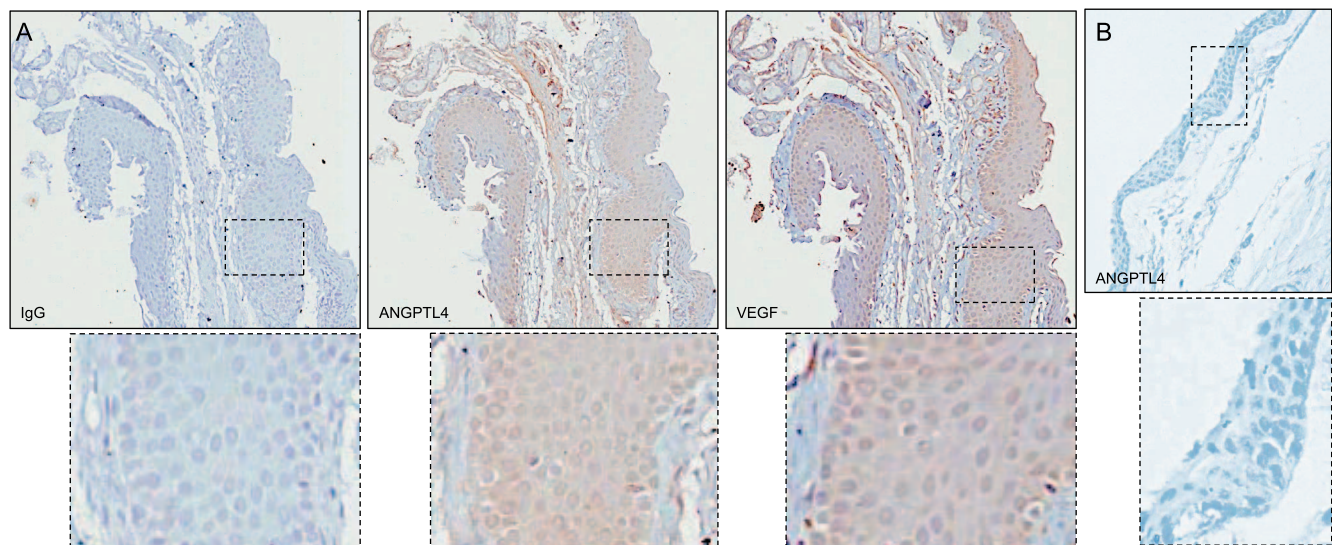


FIGURE 5. ANGPTL4 expression is detected in conjunctival epithelium from surgically excised pterygia. (A) Immunohistochemical staining of pterygia for ANGPTL4 and VEGF. IgG was used as a negative control. High-resolution insets are shown below. Similar results were obtained in 6/6 pterygia. (B) Immunohistochemical staining of ANGPTL4 in normal conjunctiva of autopsy eyes with no known history of anterior segment disease. A high-resolution inset is shown below. Similar results were observed in 4/4 autopsy eyes.

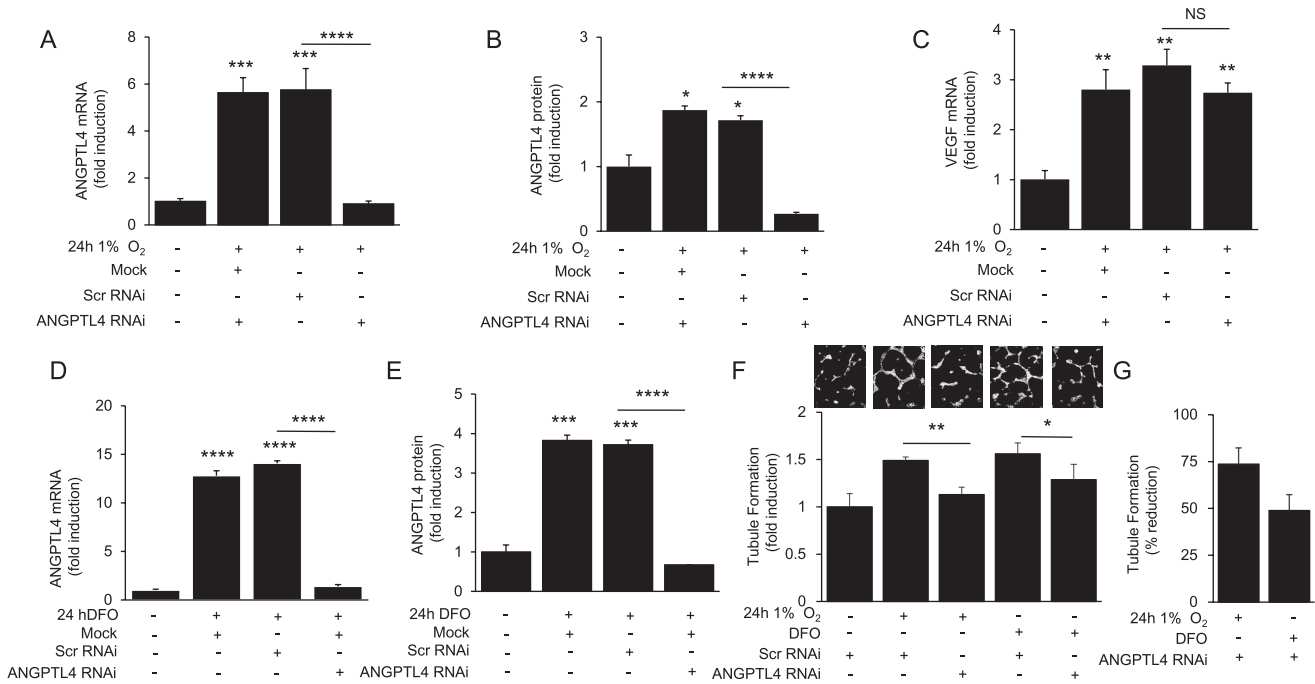


FIGURE 6. Inhibition of ANGPTL4 expression by CjECs reduces their angiogenic potential. (A, B) ANGPTL4 mRNA expression (A) and protein secretion (B) in ihCjECs transfected with RNAi targeting ANGPTL4 and exposed to 1% O₂ for 24 hours. (C) VEGF mRNA expression in ihCjECs transfected with RNAi targeting ANGPTL4 and exposed to 1% O₂ for 24 hours. (D, E) ANGPTL4 mRNA expression (D) and protein secretion (E) in ihCjECs transfected with RNAi targeting ANGPTL4 and exposed to 100 μM DFO for 24 hours. (F, G) EC tubule formation by HMVECs treated with conditioned media from ihCjECs pretreated with RNAi targeting ANGPTL4 and exposed to 1% O₂ or 100 μM DFO for 24 hours reported as fold induction (F) or percent reduction (G).

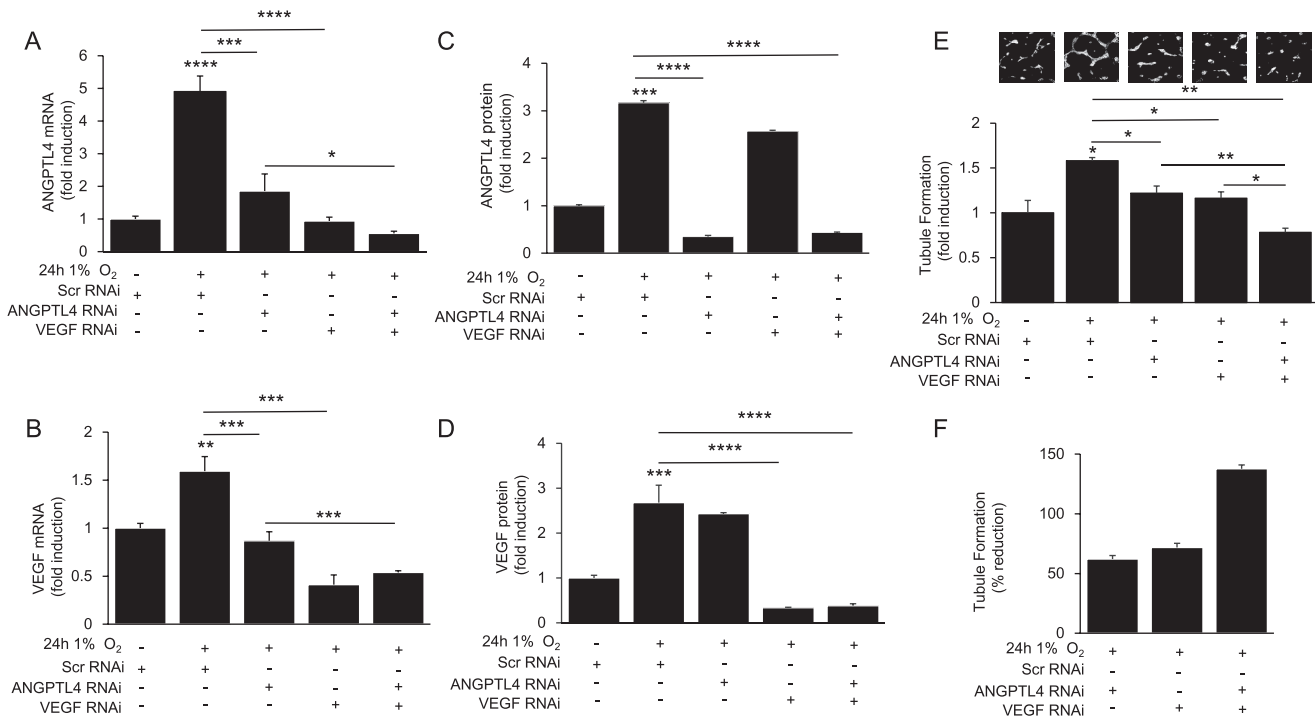


FIGURE 7. Combined inhibition of both VEGF and ANGPTL4 expression by CjECs abolishes their angiogenic potential. (A-D) ANGPTL4 (A, C) and VEGF (B, D) mRNA expression (A, B) and protein secretion (C, D) in ihCjECs transfected with RNAi targeting ANGPTL4, VEGF, or both and exposed to 1% O₂ for 24 hours. (E, F) EC tubule formation by HMVECs treated with conditioned media from ihCjECs pretreated with RNAi targeting ANGPTL4, VEGF, or both and exposed to 1% O₂ for 24 hours reported as fold induction (E) or percent reduction (F).

left untreated. There are currently no reliable medical treatments to prevent or even reduce the progression of pterygia. Consequently, aside from avoiding environmental risk factors (e.g., ultraviolet and infrared radiation from sunlight, human papillomavirus infection, trauma, inflammation, and exposure to local irritants including sand and wind)³⁷ in vulnerable populations, little else can be offered to patients to help prevent the development or progression of pterygia. Topical treatments (e.g., artificial tears, decongestants, nonsteroidal anti-inflammatory drugs, and steroids) may provide comfort and symptomatic relief for irritation and inflammation in some patients. However, definitive treatment requires surgery.^{2,38} Even with surgery prevention of recurrence remains a challenge. The recurrence rates for simple surgical excision is variable, but high, ranging from 50% to over 90%; this rate has been reduced to less than 10% in some studies with the introduction of amniotic membranes or conjunctival autografts.³⁹ More advanced surgical techniques and the addition of adjuvant therapies (e.g., β -radiation, mitomycin C, and 5-fluorouracil) has further reduced this high rate of recurrence to under 5% to 10%.³⁹ With the growing appreciation for the roles of inflammation and angiogenesis on recurrence rates, conjunctival grafting and/or the use of amniotic membranes, in combination with antiproliferative, antifibrotic, and/or anti-inflammatory approaches are becoming the standard of care to help reduce the rate of recurrences. Nonetheless, preventing recurrences remains a challenge, and adjuvant medications can themselves be associated with an increased risk for significant morbidity as a result of their adverse effects.^{39,40}

Recent reports demonstrating the expression of VEGF in pterygium tissue have encouraged clinicians to extend the use of anti-VEGF therapy to the treatment of pterygia with the goal of inducing regression of the fibrovascular component of the pterygium, thereby reducing or preventing growth.¹⁹ While preliminary evidence suggested that local treatment with anti-VEGF therapy may be effective in the treatment of ocular surface neovascularization in preclinical models, the impact of this approach in the clinic have been less impressive; most patients have only a partial response and the effects are often transient.¹³ In patients with pterygia, the use of anti-VEGF therapy has similarly met with unimpressive results.¹⁹ It remains unclear whether the limited efficacy of anti-VEGF therapy for the treatment of pterygia is because antiangiogenic therapies are simply not an effective approach for the treatment of pterygia, or if therapies targeting only VEGF are not sufficient to adequately prevent the angiogenic phenotype of pterygia. If the latter is true, then identifying and targeting additional angiogenic factors may be required to provide an effective antiangiogenic approach for the treatment of pterygia.

The identification of additional angiogenic mediators in proliferative retinopathies has been successful, in part, due to early recognition that VEGF expression is increased in ischemic retinal disease due to increased accumulation of the transcription factor, HIF-1 α . However, hypoxia is not known to play a major role in the pathogenesis of pterygia. Rather, inflammation, oxidative stress, and DNA damage are believed to be driving forces for pterygia development.³⁷ Interestingly, these same factors have also been shown to lead to the accumulation of HIF-1 α .^{9,10} Motivated by these observations, we set out to determine whether HIF-1, and in turn other HIF-regulated angiogenic mediators, contribute to the angiogenic phenotype of pterygia.

Here we demonstrate that HIF-1 α expression is increased in conjunctival epithelium from surgically excised pterygia compared to normal controls, in a similar pattern as the expression of VEGF. This observation supports a role for CjECs in promoting the vascular component of pterygia by elaborat-

ing HIF-regulated angiogenic mediators. We propose that reduced conjunctival vasculature and relative ischemia,¹¹ in combination with oxidative stress⁴¹ and inflammation, may together promote the accumulation of HIF-1 α in CjECs, triggering the angiogenic phenotype characteristic of pterygia.

Indeed, we demonstrate that accumulation of HIF-1 α in cultured CjECs results in an increase in their angiogenic potential, while inhibition of HIF-1 α accumulation blocks this effect. HIF-1 α accumulation in CjECs results in an increase in VEGF mRNA and protein expression. However, inhibition of VEGF mRNA expression using RNAi blocked expression of VEGF protein but only partially inhibited the angiogenic phenotype of these cells. Collectively, these observations support the role of additional HIF-regulated mediators in the promotion of the angiogenic phenotype of pterygia.

In this regard, we have recently reported that expression of a second HIF-regulated angiogenic factor, ANGPTL4, is increased in diabetic eye disease.^{25,27,34} ANGPTL4 is a member of the angiopoietin family of secreted proteins, which includes several members that have been reported to be critical factors in vascular development. Angiopoietin-1 (ANGPT-1) and angiopoietin-2 (ANGPT-2) participate in the development of vascular permeability by binding the TIE-2 EC receptor.⁴² While ANGPT-1 promotes vessel maturation, ANGPT-2 has been reported to antagonize the effect of ANGPT1 on vessel stabilization. Unlike ANGPT-1 and -2, ANGPTL4 does not bind to the TIE-2 receptor. ANGPTL4 has been reported to instead modulate the disposition of circulating triglycerides.⁴³ The role of ANGPTL4 in vascular biology is less clear.

Initial studies on the role of ANGPTL4 in cancer demonstrated that this cytokine may be antiangiogenic and inhibit vascular permeability, while more recent reports suggest that ANGPTL4 may instead be proangiogenic and promote permeability.⁴⁴ The conflicting roles of ANGPTL4 in angiogenesis extend to its function in the eye where ANGPTL4 has been reported to be both pro- and antiangiogenic and both pro- and antipermeability. Examination of the eyes of homozygous *Angptl4* null mice suggests that ANGPTL4 may indeed be proangiogenic.⁴⁵ We have recently observed that ANGPTL4 potently induces angiogenesis in vitro and in vivo in models of ischemic retinal disease, and can promote anterior segment neovascularization in rodents.²⁵⁻²⁷

We report here that HIF-1 α accumulation in CjECs promotes ANGPTL4 mRNA expression and protein secretion. We further demonstrate that ANGPTL4 expression is observed in the conjunctival epithelium of surgically excised pterygia. Inhibition of ANGPTL4 expression is sufficient to inhibit the angiogenic phenotype of these cells, similar to inhibiting VEGF. Collectively, these data suggest that ANGPTL4 may also contribute to the angiogenic phenotype of pterygia. These observations provide an explanation for why anti-VEGF monotherapy has not been sufficient as an antiangiogenic approach for the treatment of patients with pterygia. Instead, our findings suggest that pharmacotherapy independently targeting VEGF and ANGPTL4, or targeting HIF-1 to inhibit both, may be a more effective antiangiogenic treatment for patients with pterygia.

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