Short Term Effect of Pre-Operative Anti-VEGF on Angiogenic and Fibrotic Profile of Fibrovascular Membranes of Proliferative Diabetic Retinopathy

Kaveh Fadakar,[1,2] Safa Rahmani,[1] Thomas Tedeschi,[1] Jeremy A. Lavine,[1] and Amani A. Fawzi[1]

[1]Department of Ophthalmology, Feinberg School of Medicine, Northwestern University, Chicago, Illinois, United States
[2]Farabi Eye Hospital, Tehran University of Medical Sciences, Tehran, Iran

Correspondence: Amani A. Fawzi, Department of Ophthalmology, Feinberg School of Medicine, Northwestern University, 259 E Erie Street, Suite 1520, Chicago, IL 60611, USA;
afawzimd@gmail.com.

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Purpose. Adjuvant, pre-operative intravitreal anti-vascular endothelial growth factor (anti-VEGF) injections have been used to reduce peri-operative bleeding in eyes undergoing pars-plana vitrectomy for complications of proliferative diabetic retinopathy (PDR). To address the concern over their potential off-target effects of progressive fibrous contraction, we sought to dissect the transcriptional changes in the surgically extracted fibrovascular membranes (FVMs).

Methods. We analyzed surgically extracted FVMs from 10 eyes: 4 eyes pretreated with intravitreal bevacizumab (IVB) and 6 untreated eyes. FVMs were digested into single cells, mRNA was extracted from endothelial cell-enriched (microbead selection with CD31) and non-endothelial cell compartments, followed by RT-qPCR quantification. We then compared the relative expression of genes involved in angiogenesis, endothelial cell integrity, and myofibroblastic processes between treated and untreated FVMs.

Results. Endothelial cells from IVB pretreated FVMs showed significant reduction of *VEGFA*, VEGF receptors (*FLT1* and *KDR*), and angiopoietin 2 expression as well as increased vascular endothelial cadherin and endothelin, suggesting reduced angiogenesis and enhanced vascular integrity. The non-endothelial cell fraction showed decreased expression of *VEGFA* and fibronectin, without significant difference in the expression of other profibrotic factors.

Conclusions. Our findings confirm that adjuvant pre-operative IVB decreased fibronectin and increase endothelin-1 expression without affecting other profibrotic gene expression, uncovering an important interaction between IVB and endothelin-1 that deserves further study.

Keywords: diabetic retinopathy (DR), quantitative polymerase chain reaction (q-PCR), fibrovascular membrane (FVM), vascular endothelial growth factor (VEGF), endothelins

Proliferative diabetic retinopathy (PDR) represents an advanced stage of diabetic retinopathy (DR), and affects 7% of patients with diabetes.[1] Retinal ischemia, the main underlying pathology in PDR, mediates vascular endothelial growth factor (VEGF) production, with proliferation of aberrant new vessels along the vitreoretinal interface in advanced PDR.[2] Left untreated, inflammatory mediators and growth factors contribute to cellular proliferation and accumulation of extracellular matrix, resulting in pre-retinal fibrovascular membrane (FVM) formation.[3] FVMs are the main precursors for tractional retinal detachments (TRDs) and other complications of PDR, such as non-clearing vitreous hemorrhage (NCVH),[4] which commonly require surgical interventions. During surgery, the FVMs are dissected to release the traction on the retina, where hemostasis is an important aspect of successful surgery.

To reduce hemorrhagic complications during surgery for PDR, adjuvant pre-operative injection of anti-VEGF agents has been suggested. A body of anecdotal and cohort studies has suggested the potential benefits of pre-operative intravitreal bevacizumab (IVB), a full-length humanized monoclonal antibody against all isoforms of VEGF,[5] in lowering the risk of intra-operative bleeding during surgery.[6] Pre-operative IVB has also been anecdotally associated with rapid progression of fibrosis as a result of FVM contraction.[7] This "angiofibrotic switch" has been proposed to suggest an imbalance between VEGF-associated angiogenesis and the profibrotic activity of connective tissue growth factor (CTGF),[8,9] although the mechanistic role of IVB in this process remains to be elucidated. Accumulating evidence suggest that IVB could change the biologic microenvironment within the eye based on cytokine profile and growth factor changes in aqueous and vitreous fluid in eyes with PDR.[10–14]

Studies evaluating the effect of IVB on FVM in PDR are limited to semiquantitative immunohistologic methods,[15–20] leaving an important gap in our understanding of the biological effects of IVB on cell-specific relative gene expression of angiogenesis and fibrosis, the major pathologic drivers of FVM formation. Understanding the response to treatment on

a cellular level may facilitate the development of therapies that address pathways potentially disrupted by anti-VEGF. To achieve this goal, we examined the short-term effect of IVB on the gene expression profile of surgically extracted FVMs. We separately evaluated the endothelial cells on one side and all the other cellular elements in FVM, with a focus on angio-fibrotic response.

Methods

Patients and Procedures

Patients with PDR who presented to the retina clinic with complications that required pars-plana vitrectomy (PPV) were recruited from February 2019 to November 2022. The Institutional Review Board of Northwestern University reviewed and approved the study design and deemed this study as with minimal risk, not requiring an informed consent. All the procedures were in accordance with Declaration of Helsinki for research involving human subjects.

Inclusion criteria were the presence of PDR-related FVM, along with vision threatening complications, such as NCVH or TRD (Supplementary Table S1). All patients had received panretinal photocoagulation (PRP) for PDR at some point before surgery. Use of pre-operative IVB was done according to surgeon preference. The excised membranes were then divided into 2 groups: those obtained from eyes that received 1.25 mg IVB (bevacizumab; Genentech, Inc., South San Francisco, CA, USA) prior to surgery (IVB pretreated), and the control group that did not receive any anti-VEGF in the preceding 3 months. Patients in the IVB pretreated group received pretreatment within 5 days prior to surgery, except for one patient who underwent repeat vitrectomy for recurrent NCVH following the first surgery. The membrane was obtained during the second surgery, 28 days after IVB pretreatment. The gene expression result for this eye was within 75% percentile of the data, except for *ANGPT1* and *ANGPT2*. Based on these considerations, we decided to keep the results generated from this eye in the study. We excluded membranes extracted for pathologies other than PDR, eyes with combined rhegmatogenous and tractional retinal detachment, or history of intravitreal steroid injection.

All patients underwent a thorough ophthalmic examination. The severity of DR was evaluated using standardized color photographs. Pre-operative ultrasonography was performed if the fundus examination was precluded by vitreous hemorrhage (VH). One of three surgeons performed standard 23 or 25-gauge PPV (authors S.R. and J.L. treated eyes in the IVB groups, and author A.F. treated eyes in the control group). During surgery, the FVM were collected using the delamination technique and submitted for further analysis.

Processing of Surgical Membranes

Surgically dissected FVMs were placed in balanced salt solution (BSS) and stored on ice for immediate transportation to the laboratory. For dissociation, membranes were transferred to a 500 µL solution of 0.22 um filter sterilized 1× Dulbecco's Phosphate Buffered Saline (DPBS) with calcium and magnesium (Life Technologies, Carlsbad, CA, USA) containing 2 mg/mL Collagenase Type I (Worthington, Lakewood, NY, USA). Samples were incubated at 37°C for 30 minutes with gentle trituration every 10 minutes. Samples were then spun down at 400 g for 8 minutes at 4°C and cell pellets were resuspended in 500 µL 1× DPBS without calcium and magnesium (Life Technologies, Carlsbad, CA, USA), 1% bovine serum albumin (BSA; citation), and 2 mM EDTA and stored on ice until further processing. Because the membrane size varied from patient to patient, cell counts were not always robust enough to permit processing of genetic profiling and/or immunofluorescent techniques listed below from every sample.

Endothelial Cell Isolation and RNA Purification

A rat-anti-mouse CD31 antibody (Invitrogen, Carlsbad, CA, USA) was bound to sheep-anti-rat Dynabeads (Invitrogen, Carlsbad, CA, USA) following the manufacturer's instructions. Then, 10 µL of the Dynabead complex was added to the single cell suspension and incubated at 4°C for 1 hour with gentle rotation. Cells bound to the CD31-Dynabead complex were pulled down with a magnet and the supernatants containing unbound cells were collected. Cell supernatants were pelleted at 400 g for 8 minutes at 4°C. CD31-enriched cell pellets were washed 3 times with 1× DPBS. All cell pellets were lysed in RNA Stat-60 solution (Tel Test B Labs, Alvin, TX, USA) overnight at −80°C. Nucleic acids were isolated with chloroform, precipitated with 100% ethanol, and washed with 70% ethanol. DNAse reaction was performed following the manufacturer's suggested protocol (Thermo Scientific, Waltham, MA, USA). RNA was extracted with Phenol/Chloroform (Ambion, Austin, TX, USA) and precipitated overnight in 100% ethanol/160 mM ammonium acetate/0.8% glycogen at −80°C. RNA pellets were resuspended in 10 µL nuclease free ddH2O.

cDNA Synthesis and RT-qPCR

The cDNA was made with a SuperScript III First-Strand kit following the manufacturer's instructions (Invitrogen, Carlsbad, CA, USA). See Supplementary Table S2 for all human primer sequences. Then, 2 ng of cDNA was used as a template for RT-qPCR, which was performed using PowerUp SYBR Green Master Mix (Applied Biosystems, Foster City, CA, USA) following the manufacturer's instructions. RT-qPCR was performed on a QuantStudio3 system (Applied Biosystems, Foster City, CA, USA). Thermal cycling conditions consisted of 50°C for 2 minutes followed by an initial denaturation step at 95°C for 10 minutes and then 40 cycles of 95°C for 15 seconds and 60°C for 1 minute. A melt curve stage was used for quality control assessments. Relative gene expression between samples was used following standard 2^{-ddCt} methods. Because mRNA contamination and inadequate cell separation could be a source of bias, we first sought to confirm that our technique achieved sufficient endothelial cells enrichment. To do this, we compared the expression of genes which are either specific or have uneven expression in each compartment. Next, we compared the relative level of gene expression in endothelial cells and non-endothelial cells samples, which were separately normalized to *GADPH* gene to evaluate the response to anti-VEGF treatment. We measured each transcript in each sample twice (technical duplicates), and the average value was used for further analysis. According to the "Minimum Information for Publication of Quantitative Real-Time PCR Experiments" (MIQE) guidelines, validating the stability of the reference gene is a crucial prerequisite for RT-qPCR studies.[21] Therefore, we evaluated the relative expression of GAPDH normalized to

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βactin, which has been previously demonstrated to be a reliable reference gene in patients with diabetes. See Supplementary A1 for this analysis of housekeeping genes.

Immunohistochemistry and Imaging

After surgical removal of the PDR membrane, the tissue was fixed in 4% paraformaldehyde/PBS for 2 hours at room temperature and processed for staining. Anti-FoxO1 (C29H4) antibody (Cell Signaling Technology, Danvers, MA, USA) was incubated at 1:100 dilution, overnight, at 4°C and anti-rabbit antibody conjugated to Alexa 488 (Jackson Immunoresearch, West Grove, PA, USA) at 1:200 dilution for 2 hours and stained with NucBlue Fixed cell probe DAPI (Thermo Fisher Scientific, Waltham, MA, USA) to stain nuclei. Membranes were mounted and imaged using the Nikon W1 Dual CAM spinning Disk at the Center for Advanced Microscopy, Northwestern Feinberg School of Medicine. All images were processed in the same manner using ImageJ and Photoshop. In order to quantify the immunohistochemical (IHC) results, we measured fluorescent intensity within the endothelial cell nuclei. Using ImageJ, the DAPI stained nucleus was manually encircled, and the intensity of FOXO1 fluorescence was measured to compare the IVB versus non-injected control samples.

Statistical Analysis

Data analysis and representation was performed using Graphpad prism version 9.0.0 (GraphPad Software Inc, San Diego, CA, USA). Quantitative data are presented as mean ± standard deviation, and qualitative data as number and percentage. The Smirnov-Kolmogrov test was used to analyze the normal distribution of each variable. Independent sample *t*-test was used to compare data with normal distribution and the Mann-Whitney test to compare nonparametric variables. To evaluate the association of desired variables with normal distribution, the Pearson correlation test was used. The Spearman Rho test was used to evaluate the correlation of nonparametric variables. Any *P* value less than 0.05 was considered as significant.

Results

Baseline Characteristics

Ten eyes of 10 patients with PDR who required PPV for TRD or NCVH were included in this study. Four eyes received IVB pretreatment, between 2 and 28 days before surgery. The Table outlines the baseline demographics of the participants. No significant difference was observed regarding gender, age, duration of diabetes, and body mass index (BMI) between the two groups.

Endothelial Cell Enrichment

To confirm successful endothelial enrichment, we compared the relative endothelial versus non-endothelial expression of genes with known specific or predominant expression in the different cellular compartments (Fig. 1). As expected, relative expression of *FLT1* and *CDH5* were enriched in the endothelial cells (see Figs. 1A, 1B), whereas *ANGPT1* and *TGFβ* had greater expression in the non-endothelial cells compartment (see Figs. 1C, 1D).

Expression of Angiogenetic Factors

We then investigated the effects of IVB treatment on angiogenic factors and their receptors within each cellular compartment. Pretreatment with IVB resulted in significant reduction of *VEGFA* expression in both the endothelial cell (1.04 ± 0.18 vs. 0.009 ± 0.009, $P < 0.001$) and the non-endothelial cell compartments (1.15 ± 0.60 vs. 0.04 ± 0.01, $P = 0.007$; Figs. 2A, 2C). We also found significantly downregulated expression of VEGFR1 (*FLT1*; 1.04 ± 0.17 vs. 0.40 ± 0.22, $P = 0.001$), VEGFR2 (*KDR*; 1.04 ± 0.18 vs. 0.05 ± 0.07, $P < 0.001$), and *ANGPT2* (1.19 ± 0.44 vs. 0.25 ± 0.35, $P = 0.008$) in IVB pretreated endothelial cells but not in the non-endothelial cell fraction (see Figs. 2A, 2C). *ANGPT1* relative gene expression did not differ in either compartment (1.02 ± 0.19 vs. 0.87 ± 0.37, $P = 0.415$ and 1.13 ± 0.60 vs. 1.25 ± 0.28, $P = 0.717$; in endothelial cells and non-endothelial cells, respectively; see Figs. 2A, 2C).

We next investigated the effects of IVB pretreatment on the tight junction protein VE-Cadherin (*CDH5*) and the endothelin system. We observed a significant increase in the expression of *CDH5* and *EDN1* in IVB pretreated endothelial cells (1.05 ± 0.32 vs. 3.83 ± 1.60, $P = 0.003$ and 1.22 ± 0.78 vs. 6.46 ± 2.38, $P = 0.001$; respectively; see Fig. 2B). However, the expression of the endothelin receptors ETA (*EDNRA*) and ETB (*EDNRB*) were not significantly different (see Fig. 2B). In the non-endothelial compartment, we found no significant alteration in relative gene expression of *CDH5*, *EDN1*, *EDNRA*, or *EDNRB* (see Fig. 2D).

To investigate whether *ANGPT1* and *ANGPT2* were co-regulated with *VEGFA* and its receptors, we evaluated their correlation (Fig. 3). A significant, moderate to strong correlation was observed between *ANGPT2* and *VEGFA* ($r = 0.788$, $P < 0.001$; see Fig. 2D), *FLT1* ($r = 0.804$, $P < 0.001$; see Fig. 3E), and *KDR* ($r = 0.874$, $P < 0.001$; see Fig. 3F). In contrast, the association of *ANGPT1* with *FLT1* and *KDR* was moderate to weak ($r = 0.502$, $P = 0.024$ and $r = 0.546$, $P = 0.013$, respectively), and it was not correlated with *VEGFA*.

We next evaluated the potential mechanism driving increased *EDN1* expression following IVB injection. Forkhead box protein O1 (FOXO1), a transcription factor down-

Table. Comparison of Clinical and Baseline Characteristics of Treated Versus Control Group

Clinical Character	IVB Treated ($n = 4$)	Control ($n = 6$)	*P* Value
Female (%)	1 (25)	4 (66.6)	0.524
Age, y	51.25 ± 7.88	54.83 ± 14.09	0.660
Duration of diabetes, y	20.25 ± 14.59	20.50 ± 5.44	0.975
HbA1C (%)	7.60 ± 0.94 (60 mmol/mol)	8.13 ± 1.97 (65 mmol/mol)	0.633
BMI (kg/m^2)	35.01 ± 5.21	29.40 ± 4.18	0.095

BMI, body mass index; HbA1C, hemoglobin A1C; IVB, intravitreal bevacizumab.

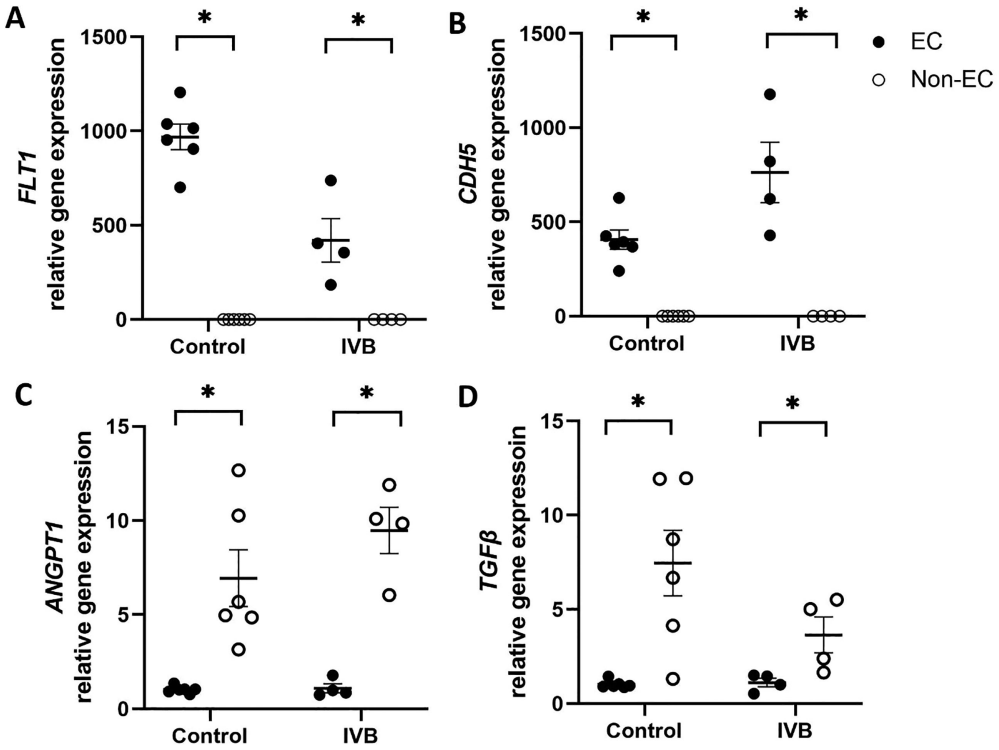

Figure 1. Relative expression of genes with known endothelial cells specificity. To confirm endothelial enrichment, expression of genes with known specificity for each compartment were evaluated. *FLT1* and *CDH5* were selected as endothelial specific genes. *ANGPT1* and *TGFβ* were expected to have uneven expression toward higher expression in non-endothelial compartment. In both control and treated samples, expression of *FLT1* and *CDH5* were significantly higher in endothelial cells compared to non-endothelial cells, confirming enrichment (**A, B**). *ANGPT1* and *TGFβ* were significantly higher in the non-endothelial compartment, as expected (**C, D**).

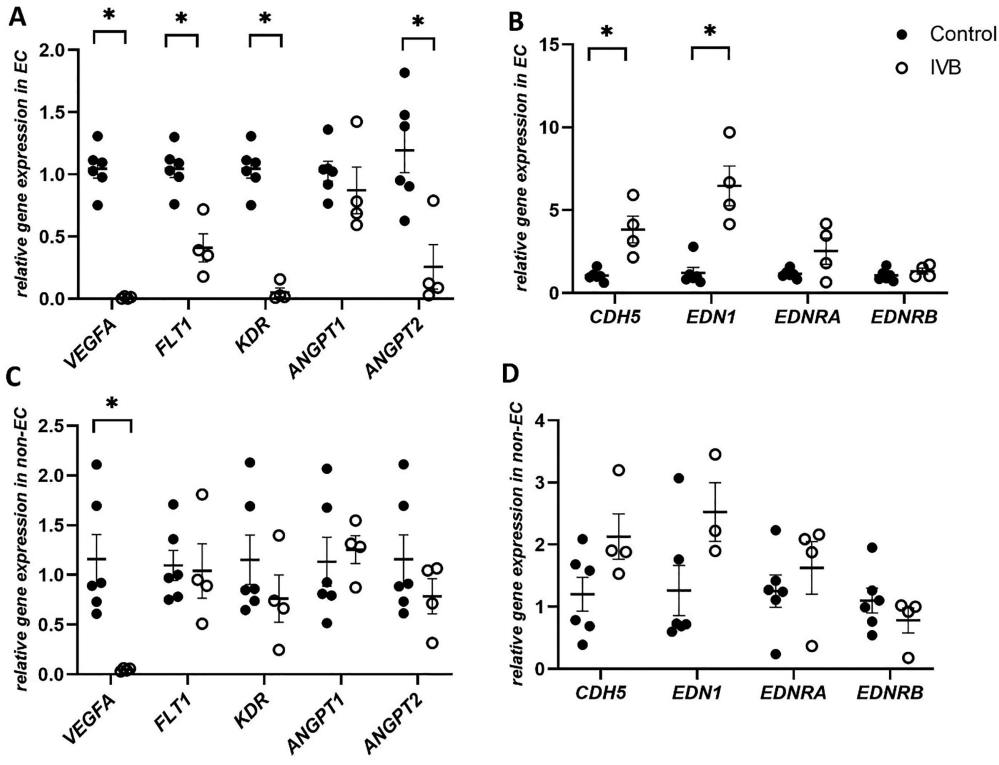

Figure 2. Alteration of various angiogenic factors following preoperative intravitreal bevacizumab (IVB). In endothelial cells (ECs), IVB results in significant reduction of relative gene expression of *VEGFA*, *FLT1*, *KDR*, and *ANGPT2* (**A**), accompanied by an increase in relative gene expression of *CDH5* and *EDN1* (**B**). In the non-endothelial cells (non-ECs), only *VEGFA* relative gene expression was decreased significantly (**C, D**).

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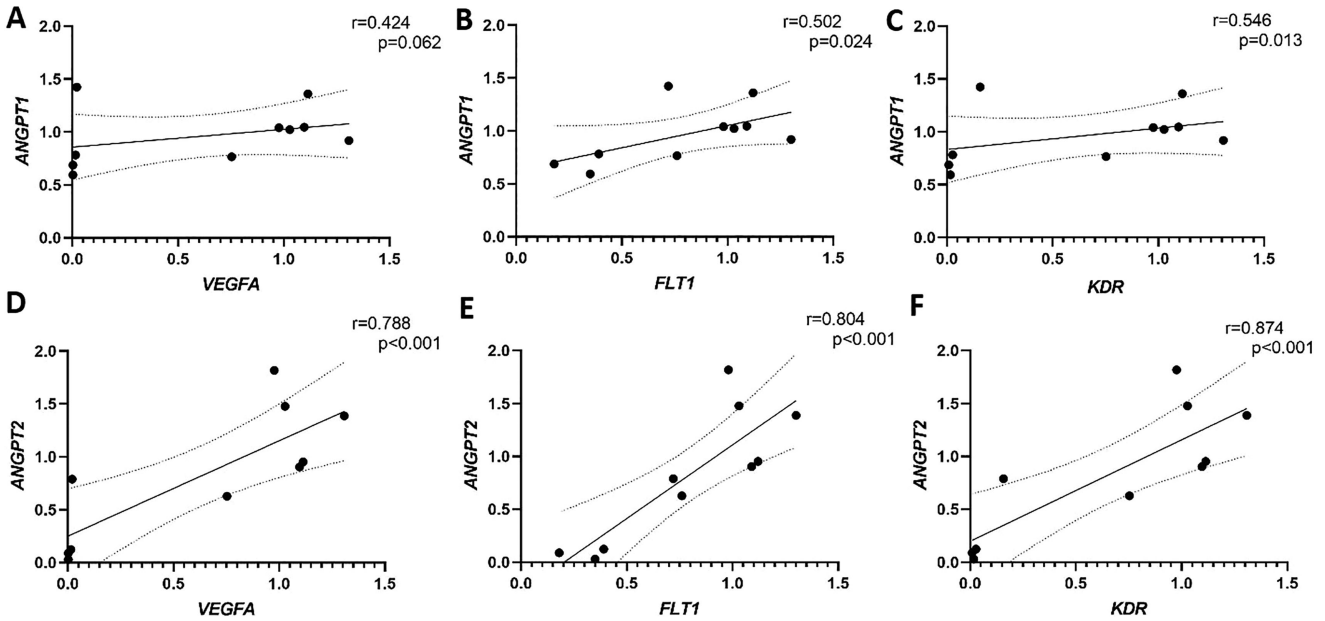

Figure 3. Association of *ANGPT1* and *ANGPT2* with *VEGFA* and its receptors. *ANGPT1* does not have a significant association with *VEGFA*. Although *ANGPT1* was significantly correlated to *FLT1* and *KDR*, the strength of the association was moderate to weak ($r = 0.0502$ and 0.546, respectively). In contrast, *ANGPT2* demonstrated significant, moderate to strong correlation with *VEGFA* and its receptors.

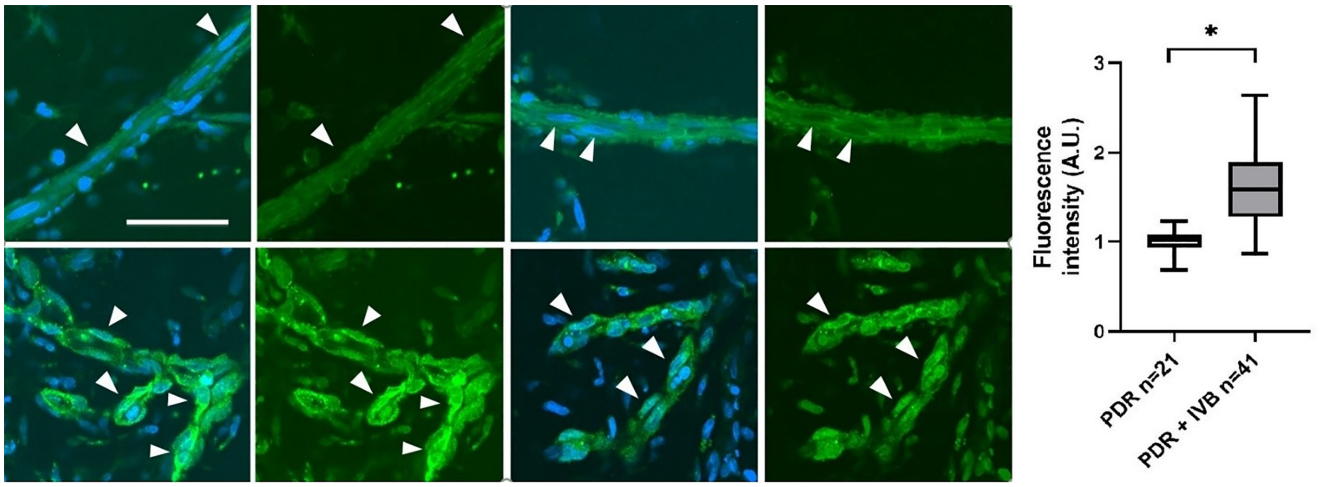

Figure 4. Nuclear localization of FOXO1 after intravitreal bevacizumab (IVB) injection. Flatmount of two different proliferative diabetic retinopathy fibrovascular membranes; the *top panel* shows an untreated sample and bottom panel is the IVB-injected. *Arrow heads* point to relative nuclear localization of FOXO1 (*green*) after IVB injection (*lower panel*), compared to the untreated sample (*upper panel*). Nuclei are labeled with DAPI (*blue*). The panel to the *right* shows the quantitative assessment of FOXO1 fluorescence intensity in the nucleus. Following bevacizumab injection, FOXO1 showed significantly higher fluorescence intensity in endothelial nuclei (n = endothelial cells). Scale bar = 50 µm.

stream of the VEGFR2 pathway, is a major regulator of *EDN1* transcription. FOXO1 is activated by phosphorylation, leading to its translocation from the cytoplasm into the nucleus, where it initiates transcription of downstream effectors. We hypothesized that interruption of VEGFR2 signaling by IVB shuttles FOXO1 into the nucleus, which would explain the increased transcription and expression of *EDN1*. As shown in Figure 4, we confirmed FOXO1 in endothelial cells of IVB treated FVM showed significantly higher nuclear localization than untreated endothelial cells (see Fig. 4).

Expression of Myofibroblastic Products and Profibrotic Factors

Figure 5 demonstrates the relative gene expression of myofibroblastic products and profibrotic factors. Expression of fibronectin (*FN1*) was significantly reduced in the non-endothelial cell fraction following IVB pretreatment (1.42 ± 0.85 vs. 0.20 ± 0.37, $P = 0.030$; see Fig. 4C). No significant changes were observed in the expression of other genes (see Fig. 5).

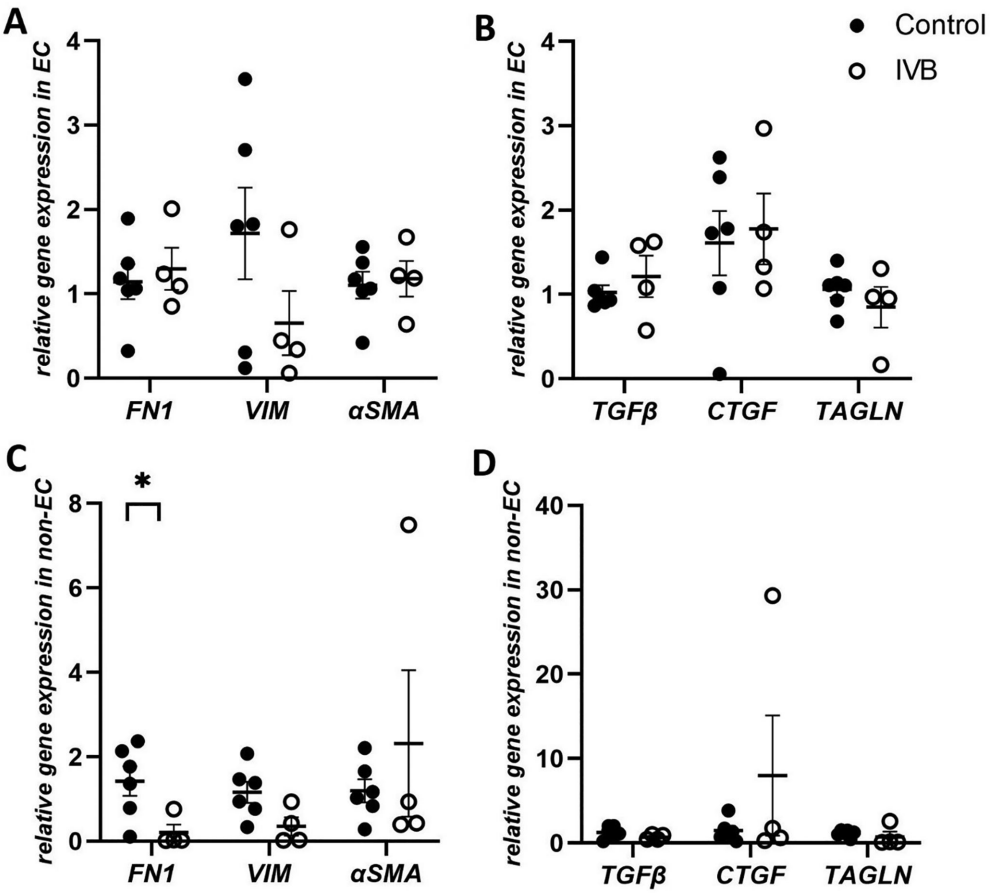

Figure 5. Alterations of myofibroblastic products (**A, C**) and profibrotic factors (**B, D**) following pre-operative intravitreal bevacizumab (IVB) injection. Relative gene expression of fibronectin (*FN1*) was decreased significantly in the non-endothelial cells (non-ECs) (**C**) without significant alteration of other factors (**C, D**). None of the factors changed significantly in endothelial cells (ECs) (**A, B**).

Discussion

In this study, we evaluated the short-term effects of anti-VEGF on the gene expression profiles in FVM extracted during surgical repair of advanced PDR-related complications. We measured angiogenesis and fibrotic gene expression in endothelial and non-endothelial cellular compartments separately. We observed that IVB effectively downregulated endothelial expression of *VEGFA*, *FLT1*, *KDR*, and *ANGPT2*, indicating suppression of angiogenesis and improved vascular stability, as expected. We also observed a potential cross talk between *ANGPT2* and *VEGFA* signaling, notably as a strong correlation between *ANGPT2* and *KDR*. As well, we report markers of improved endothelial junction integrity and vasoconstriction as indicated by increased expression of *CDH5* and *EDN1*. Our finding revealed a previously unrecognized increased *EDN1* expression after IVB. We show nuclear localization of FOXO1 in IVB injected FVM, which we propose as the mechanistic driver for increased transcription of the *EDN1* gene (see Figs. 4, 6). Unexpectedly, we found no increase in fibrosis signaling in IVB-injected FVM samples, instead, we found significantly decreased fibronectin expression (see Fig. 5).

Unexpectedly, we found that IVB pretreatment does not appear to exacerbate the fibrotic response within the cellular compartments of the FVM in the short term, instead, we observed relatively decreased expression of *FN1* in the non-endothelial compartment. Further, other myofibroblastic products, such as *VIM* and *αSMA*, and profibrotic cytokines, such as *TGFβ*, *CTGF*, and *TAGLN*, were not significantly different. However, interpretation of our results should be considered with caution. Absence of transcriptional changes within the FVM tissue does not necessarily contradict the putative fibrotic reaction, which could be driven by secreted retinal cytokines in response to IVB, as has been shown in studies using vitreous samples.[8,9] Given that *TGFβ* expression is upregulated in eyes with PDR,[22–24] the lack of alteration in *TGFβ* expression following IVB may suggest unimpeded profibrotic activity. Additionally, considering the lack of altered expression of *CTGF* and reduced expression of *VEGFA*, the ultimate effect could be higher CTGF/VEGFA ratio, which has been shown to correlate with increased fibrosis.[25] This process has been termed the "angiofibrotic switch" as related to IVB treatment. In contrast to our study, Zhang et al. showed increased expression of TGFβ and CTGF in fibrovascular membrane specimens.[15] One notable difference between our study and the latter study is their focus on eyes that showed clinical evidence of progression of fibrosis. It is plausible that an altered profibrotic expression profile might be observed in a subset of PDR eyes, where it translates to intense fibrotic response. Evaluation of cell specific expression of *CTGF* and *TGFβ*, measurement of protein products in the vitreous, and direct quantification of fibrotic activity may allow us to precisely dissect the

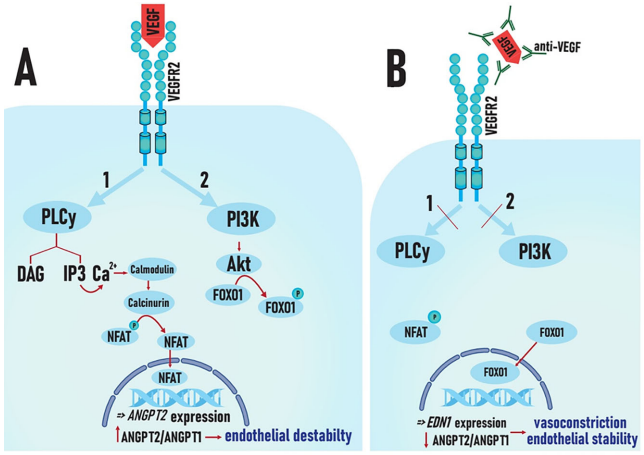

Figure 6. Proposed mechanism of altered gene expression following intravitreal bevacizumab (IVB) pretreatment. Part A shows the effect of VEGFA activation of VEGFR2 signaling. Via Ca^{2+}-calmodulin-calcineurin pathway, dephosphorylated NFAT is translocated to the nucleus, which leads to expression of *ANGPT2* (A. pathway 1). Phosphorylated FOXO1, through activation of PI3K-Akt, remains in cytoplasm (A. pathway 2). The ultimate outcome would be increased ANGPT2/ANGPT1 ratio, and vessel destabilization. Part B depicts blockage of VEGFR2 following injection of anti-VEGF. Disruption of Ca^{2+}-calmodulin-calcineurin pathway leads to decreased *ANGPT2* expression (B. pathway 1). FOXO1 remains dephosphorylated after abortion of PI3K-Akt pathway. Dephosphorylated FOXO1 shuttles into the nucleus, leading to increased transcription of *EDN1* (B. pathway 2). The outcome would be increased *EDN1* expression and decreased ANGPT2/ANGPT1 ratio, which results in vasoconstriction and vessel stabilization.

pathogenesis of this fibrosis response and the effect of IVB. Further, as shown by our recent single cell RNA study,[26] pericyte to myofibroblastic transition may be driving the fibrosis response in these PDR membranes, and the effect of IVB on this process would be an important field of further study.

We observed increased expression of both *CDH5* and *EDN1* in FVM endothelial cells after IVB injection. VE-cadherin (encoded by *CDH5*) is a specialized type II adherens junction, localized to the inter-endothelial junctions.[27] Endothelin (EDN1) is a potent vasoconstrictor and is thought to function through its receptors, ETA and ETB.[28] Accordingly, the anticipated phenotype with increased expression of *CDH5* and *EDN1* would be constricted vessels with decreased leakage, contributing to the beneficial effects of pre-operative anti-VEGF injection. This is corroborated by a recent study from our group that showed intravitreal anti-VEGF injections are associated with decreased blood flow at the deep capillary plexus,[29] in line with decreased blood flow as a consequence of vasoconstriction, highlighting the potentially complex hemodynamic consequences of anti-VEGF injections.

Our results highlight the interaction between IVB and *EDN1* in endothelial cells of the FVMs. Elevated circulating EDN1 has been reported in the setting of systemic bevacizumab therapy in renal cell carcinoma and gastrointestinal stromal tumors,[30,31] linking EDN1 mechanistically to the off-target hypertensive side effect of systemic bevacizumab. The exact mechanism underlying the relationship between VEGFR2 inhibition and *EDN1* expression remains unclear. The FOXO1, a transcription factor acting downstream of VEGFR2, is a major regulator of *EDN1* expression.[32] When phosphorylated, via activation of AKT, FOXO1 tends to remain in the cytoplasm, disabling its transcriptional function. We therefore sought to confirm the hypothesis that VEGFR2 inhibition in IVB leads to nuclear shuttling of FOXO1, potentially driving downstream *EDN1* transcription (see Fig. 6). Our finding of increased nuclear localization of FOXO1 in endothelial cells of IVB pretreated FVM confirms this hypothesis (see Fig. 6). Similar to our observation, bevacizumab-induced *EDN1* expression was associated with increased nuclear expression of FOXO1 protein in human glomerular endothelial cells.[32] To our knowledge, this has not been explored in previous studies using diabetic FVM, clarifying another potential off-target effect of IVB (see Fig. 5). Whereas systemic hypertension has been anecdotally associated with IVB, it is more likely that heightened EDN1 expression in the eye would contribute to retinal vasoconstriction and reduced retinal blood flow,[28,29] although this needs further confirmation.

Increased expression of *CDH5* following IVB could be explained by VEGFA signaling through VEGFR2. VEGFA can internalize VE-cadherin through the VEGFR2-SRC pathway, and lead to vascular leakage.[33] This process is counteracted following IVB pretreatment. Contrasting with our study, vitreous concentration of VE-cadherin has been shown to decrease significantly in eyes with PDR after treatment with IVB.[10] Although the discrepancy could be attributable to different sampling site (vitreous versus FVM) and methodology (Western blot analysis versus q-PCR), it is noteworthy that it could also be explained by IVB-induced neovascular regression. This could lead to quantitatively decreased endothelial cells, and therefore lower VE-cadherin concentration in the vitreous. In contrast, our study quantified *CDH5* expression in the remaining FVM endothelial cells. This finding is clinically reflected as lack of leakage in the large-caliber persistent PDR neovessels, with pruning of immature capillary loops in the neovessels.[34,35]

Our results showed that treatment with anti-VEGF decreased the expression of *VEGFA* in both endothelial and non-endothelial cellular components of the FVM. Interestingly, IVB reduced the expression of *FLT1* (encoding VEGFR1) and *KDR* (encoding VEGFR2) only in the endothelial cells. Previous studies have shown decreased expression of VEGF in PDR membranes following IVB.[36,37] VEGFA is a crucial survival factor of endothelial cells, mediating its effects mainly through VEGFR2. Decreased signaling through VEGFR2 could lead to endothelial cell apoptosis. This is reflected in reduced endothelial cell density in fibrovascular membrane following injection of IVB.[37] Reduction of expression of VEGFA following IVB might also reflect the endothelial VEGFA autocrine loop, thought to regulate endothelial expression of VEGFA and its receptors, in response to exogenous VEGFA.[38] In addition to its direct effects on preventing VEGF binding, disruption of this autocrine loop might be a key step in the vessel suppressive effect of anti-VEGF therapy.

Our data show that anti-VEGF therapy did not affect the expression of *ANGPT1* in either endothelial cells or non-endothelial fraction, whereas *ANGPT2* expression showed a significant reduction. It has been proposed that VEGF-independent angiogenic mediators, such as the angiopoietins, play an important role in the pathology of PDR. ANGPT1 is an important effector which regulates endothelial interactions with its supporting cells. ANGPT1 is widely expressed in perivascular cells, such as pericytes and vascular smooth muscles.[39–41] Expression of ANGPT1, mainly by

pericytes, is involved in promoting endothelial cell survival and vascular stability, through phosphorylation of the receptor TIE2.[40] *VEGFA* and *ANGPT2* are highly expressed in hypoxic conditions.[42–44] ANGPT2 has a more complex role and can function as either TIE2 agonist or antagonist, depending on the context.[45] ANGPT2 is also thought to be involved in vascular destabilization and has a putative role in pericyte detachment. It also sensitizes blood vessels to VEGFA and the loss of VE-cadherin.[46,47] Because we observed a decrease in the expression of *ANGPT2* in endothelial cells, and no alteration of ANGPT1 in either compartment, it is plausible that IVB tips the balance toward a higher ANGPT1/ANGPT2 ratio, improving vessel stabilization. This hypothesis is supported by the finding of increased expression of *CDH5*, suggesting improved tight junctions, in IVB-treated endothelial cells.

Administration of IVB leads to down regulation of both *VEGFA* and *ANGPT2* with a strong correlation between the two transcripts. We posit there could be a potential cross talk between these pathways (see Fig. 3). This finding is supported by previous studies, such as reports of increased *ANGPT2* expression in microvascular endothelial cells in response to VEGFA,[43] mediated via VEGFR2.[48] Increased expression of *ANGPT2* via VEGFR2 could be mediated through Ca^{2+} signaling and activation of nuclear factor of activated T cell (NFAT; see Fig. 6). It has been shown that calcineurin-NFAT directly activates ANGPT2 in endothelial cells of the metastatic niche.[49] It is important to note that the regulation of *ANGPT2* through VEGFA signaling could be transient, and other relevant pathways such as COX2 may be also involved.[50,51] Further studies are required to better elucidate the complex interaction of VEGFA and ANGPT2 signaling in these membranes.

Our study has some limitations. Our sample size is small, which imposes a possible β error that may have obscured small differences in relative mRNA expression. Our present observation is restricted to the short-term effects of IVB within the FVM, but did not evaluate secreted vitreous cytokines, or retinal gene expression. In addition, mRNA expression may not necessarily suggest the downstream proteins are expressed. Comparative validation of the relationship between protein synthesis and gene expression should be addressed in future studies. Further, the exact cellular source of the non-endothelial sample is largely unknown and could be attributable to several elements, including pericytes, fibroblasts, and macrophages, as we have recently shown.[26] Retinal sources and the vitreous microenvironment, which are also influenced by anti-VEGF therapy, were not evaluated in our study and could potentially shape the outcome of IVB. As well, our study only focused on a small number of effectors. Additionally, whereas the endothelial enrichment is evident from our results, possible cellular cross-contamination might distort the attribution of gene expression to specific cells. Single-cell RNA sequencing could be a more sensitive, in-depth alternative to further elucidate cell-specific expression and the effect of antiVEGF.[26]

In summary, we observed an expected significant down-regulation of angiogenic factors following IVB injection in eyes with PDR and highlight an unforeseen cross talk between VEGFA and ANGPT2. Decreased expression of *VEGFA* and *ANGPT2*, accompanied by increased expression of *CDH5* and *EDN1*, could result in suppression of pathologic angiogenesis, improved vascular stability, vasoconstriction, and overall improved endothelial integrity. Using VEGFR2 signaling as the main cellular pathway for the observed alterations, we propose a mechanistic explanation (see Fig. 6). This is supported by our findings of a strong correlation between *ANGPT2* and *KDR*, as well as increased nuclear localization of FOXO1, a VEGFR2-related transcriptional regulator of *EDN1*, in the IVB-injected samples. Further studies are needed to address the retinal cellular response to IVB as well as further define the contribution of non-endothelial components of the FVM.

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