

Vitreous levels of placental growth factor correlate with activity of proliferative diabetic retinopathy and are not influenced by bevacizumab treatment

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Abstract

Purpose Placental growth factor (PIGF) is a member of the VEGF family that plays an important role in experimental models of diabetic retinopathy and retinal neovascularization. We aimed to investigate whether vitreous levels of PIGF correlated with proliferative diabetic retinopathy (PDR) status, VEGF levels, and bevacizumab treatment. We also analysed PDR membranes to confirm the presence of the PIGF receptor, FLT1, in endothelial cells.

Methods This was a case-control study: undiluted vitreous fluid samples were obtained from 28 active PDR patients without preoperative bevacizumab treatment, 21 active PDR patients with preoperative bevacizumab treatment, 18 inactive PDR patients, and 21 control patients. PIGF and VEGF levels in samples were determined by enzyme-linked immunosorbent assay. Immunohistochemistry for FLT1 was performed on human PDR membranes.

Results Compared to control, vitreous PIGF levels were higher in both active PDR without bevacizumab ($P < 0.0001$) and with bevacizumab ($P < 0.0001$). There was no significant difference in PIGF between active PDR patients without and with bevacizumab ($P = 0.56$). Compared to active PDR, PIGF levels were significantly reduced in inactive PDR ($P = 0.004$). PIGF levels were highly correlated with VEGF levels in active PDR. VEGFR1 was expressed in endothelial cells in human PDR membranes.

Conclusion The strong correlation of PIGF levels with PDR disease status and expression of FLT1 in human PDR membranes suggest

that PIGF has a pathogenic role in proliferative diabetic retinopathy. Therapeutic targeting of PIGF with agents like aflibercept may be beneficial.

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Introduction

Diabetic retinopathy is a major cause of vision loss globally, and its global impact is expected to increase significantly due to the increasing prevalence of diabetes mellitus and the increasing life span of individuals with diabetes.¹

In Saudi Arabia, diabetes has become far more prevalent in the past two decades, with a prevalence of 23.7% in adults, ten-fold higher than in 1980.² This is associated with an overall 19.7–31.3% prevalence of diabetic retinopathy.^{3,4} Proliferative diabetic retinopathy (PDR) is the most common cause of severe visual loss in diabetes, and has an estimated world-wide prevalence of 17 million individuals.⁵ Neovascularization is the hallmark of PDR and often results in vitreous haemorrhage and tractional retinal detachment, which can lead to severe loss of vision. The identification of vascular endothelial growth factor (VEGF) as a major stimulus for PDR has led to increasing clinical management of this condition with anti-VEGF therapies. However, it is highly likely that other important angiogenic growth factors are involved in this condition,⁶ which could serve as additional targets for therapy.

A significant body of evidence in basic research and pre-clinical research has emerged

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supporting the pathogenic role of placental growth factor (PlGF). PlGF is a member of the VEGF family, and binds the VEGF receptor FLT1. In contrast to VEGF, PlGF is not required for normal health, including embryonic development.^{7,8} Similar to VEGF, PlGF is upregulated by hypoxia. Studies of PlGF knockout mice indicate that PlGF is not required for physiological angiogenesis, but does play a critical role in angiogenesis under pathological conditions.⁸ Among its cellular activities, PlGF has direct effects on vascular endothelial and mural cells in promoting angiogenesis.⁷ Of particular importance for PDR, PlGF has been found to promote retinal neovascularization in a mouse model of ischaemia-induced retinopathy.⁹ Interestingly, a recent study has also implicated PlGF in earlier diabetic retinopathy, including retinal vascular leakage.¹⁰

The clear pathogenic role of VEGF in PDR has led to the increasing use of anti-VEGF compounds in this setting, especially when there is accompanying neovascular glaucoma or vitreous haemorrhage.¹¹ Currently, three anti-VEGF drugs are in common use, bevacizumab (Avastin; Genentech Inc., San Francisco, CA, USA), ranibizumab (Lucentis; Genentech Inc.), aflibercept (Eylea; Regeneron Pharmaceuticals Inc., Tarrytown, NY, USA).¹² Although all three effectively bind VEGF and reduce its ocular levels, the three have important differences, including their structure and VEGF-binding affinity. An additional difference between the drugs is their growth factor specificity. Aflibercept in particular is a soluble fusion protein of the extracellular domain of VEGFR-1 and VEGFR-2 and the Fc region of immunoglobulin G (IgG), allowing it to efficiently bind both VEGF-A and PlGF.¹² This additional ability to bind PlGF could potentially result in differential effects of aflibercept compared to the more specific anti-VEGF drugs. On the other hand, although bevacizumab and ranibizumab are specific for VEGF, studies suggest that bevacizumab treatment can influence ocular levels of other cytokines, which may be attributable to the acute reduction in VEGF levels.^{13,14} Since VEGF is a known regulator of PlGF expression,^{15,16} it is conceivable that anti-VEGF treatment could modulate PlGF levels.

PlGF has been found to be upregulated in patients with PDR,^{17,18} suggesting that it might be involved in promoting this condition. However, further studies are desirable to support and strengthen a pathogenic role for PlGF in PDR. To this end, we collected vitreous samples from patients with and without PDR. Our objective was to evaluate PlGF in three respects—correlation with level of PDR disease activity, active *vs* quiescent; potential influence of anti-VEGF; correlation with VEGF levels. In addition, we examined the presence and localization of the PlGF receptor FLT1 in PDR membranes.

Materials and methods

Study participants

Patients undergoing vitreoretinal surgery at the King Khaled Eyes Specialist Hospital (KKESH) were recruited for the study. A total of 67 eyes of 67 patients with type 2 diabetes who were affected by PDR were enrolled in this study. The patients were divided into four groups. The eyes were classified as active PDR if there was perfused iridic or pre-retinal neovascularization visible on examination at the time of clinical examination and surgery. Based on the presence of the above features, 49 eyes were classified as active PDR, and 18 as inactive PDR. The inactive PDR cases did not receive pre-operative bevacizumab. The active PDR cases were classified according to the administration of pre-operative bevacizumab, within 7 days of surgery. The control group consisted of 21 eyes of 21 patients with no DR and control ocular conditions who underwent vitreoretinal surgery; the ocular conditions were non-angiogenic disease processes, including macular hole, rhegmatogenous retinal detachment, vitreomacular traction, and dropped nucleus. Sample sizes were chosen based on two previous studies of vitreous PlGF.^{17,18}

The study followed the tenets of the Declaration of Helsinki. The Institutional Review Boards at the Johns Hopkins School of Medicine and the King Khaled Eye Specialist Hospital approved the study protocol, and study participants gave informed consent to the treating surgeon.

Sample collection

For vitreous specimens, undiluted vitreous fluid samples were obtained at the time of vitreoretinal surgery. All patients underwent pars plana vitrectomy with either 23-gauge or 25-gauge vitrectomy technique. Undiluted vitreous samples were first obtained prior to switching the infusion line on, collected into sterile tubes, and transferred immediately to ice. Vitreous specimens were stored at -80°C and kept frozen until analysis. PDR membranes were also collected at the time of vitreoretinal surgery. The excised membranes were fixed in 10% formalin.

Measurement of vitreous PlGF and VEGF by ELISA

The levels of PlGF and VEGF in human vitreous were measured using commercial ELISA kits (Duosets from R&D, Minneapolis, MN, USA). The minimum detectable dose (MDD) was determined with an assay recommended by R&D. Specifically, we calculated MDD by adding two standard deviations to

the mean optical density of 20 zero standard replicates, then calculating the corresponding concentration from the standard curve. The MDDs for PlGF and VEGF Duosets were 3.7 and 24.8 pg/ml, respectively. The ELISA procedures were performed according to the instructions from the manufacturer.

Immunohistochemistry

Deparaffinized sections were boiled in 1 × target retrieval solution (Dako, Carpinteria, CA, USA) for 20 min. After blocking in 5% normal goat serum diluted in PBS, the sections were incubated with anti-FLT1 antibody (1 : 200, Santa Cruz, Dallas, TX, USA), anti-CD31 antibody (1 : 100, Abcam, Cambridge, MA, USA) or the isotype- and concentration-matched normal IgG control overnight at 4 °C. After three washes with PBST (PBS plus 0.1% Tween 20), sections were then incubated with the secondary biotinylated goat anti-rabbit or anti-mouse IgG (1 : 2000) for 1 h at room temperature. Sections were then detected by the alkaline phosphatase detection system (Vectastain ABC-AP kit, Vector Laboratories, Burlingame, CA, USA) and a blue reaction product was produced by incubating sections with alkaline substrate (Vector blue AP substrate kit III, Vector Laboratories). Sections were also stained with haematoxylin and eosin.

Statistical analysis

Wilcoxon sum rank test was used to compare the median of PlGF and VEGF between groups. Spearman correlation was calculated to study the correlation between the levels of VEGF and PlGF in active PDR without the bevacizumab group. Differences were considered statistically significant at $P < 0.05$.

Results

Increased vitreous levels of PlGF in PDR patients and lack of effect of bevacizumab treatment on the vitreous levels of PlGF in active PDR patients

We harvested undiluted vitreous samples from the eyes of patients with active PDR, inactive PDR and control conditions (no DR). PlGF was undetectable in the control group and assigned the value of 3.7 pg/ml, representing the limit of detection. Vitreous PlGF levels were significantly increased in both the active PDR group without bevacizumab (24.8 pg/ml, range: 3.7–773.5; $P < 0.0001$) and with bevacizumab (11.5 pg/ml, range: 3.7–211.4; $P < 0.0001$; Figure 1 and Table 1). The vitreous PlGF levels in the inactive PDR group (which did not receive pre-operative bevacizumab) were significantly reduced (11.0 pg/ml, range: 3.7–96.9; $P = 0.004$) compared to the active PDR group without bevacizumab (Figure 1 and Table 1). Vitreous PlGF levels in this inactive PDR

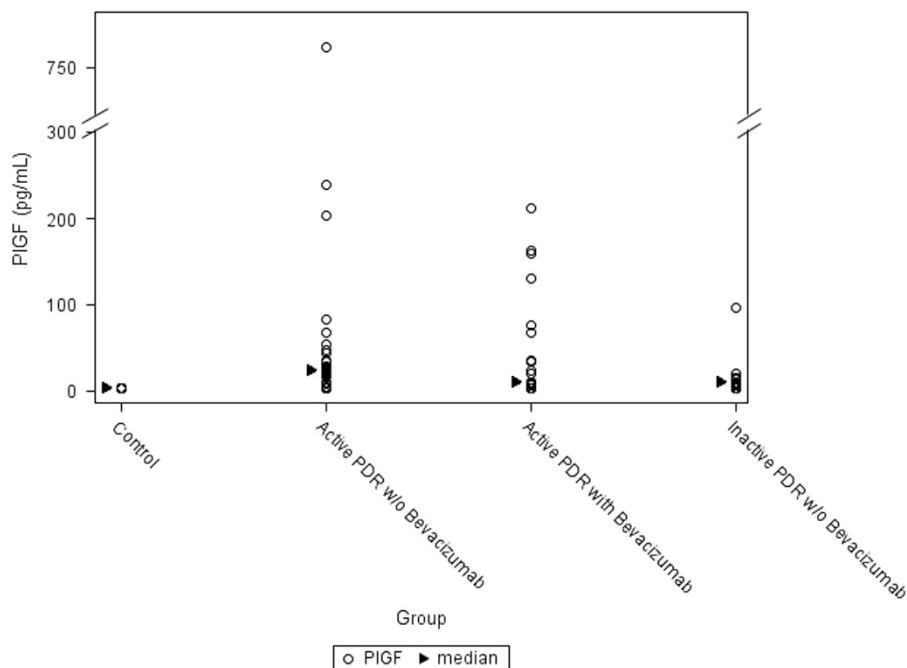


Figure 1 PlGF levels in the vitreous among active PDR without ($n = 28$) or with ($n = 21$) bevacizumab treatment, inactive PDR ($n = 18$) and no DR ($n = 21$).

Table 1 Group comparison of median of PIGF and VEGF

	No DR (control) (n = 21)	Active PDR w/o bevacizumab (n = 28)	Active PDR with bevacizumab (n = 21)	Inactive PDR (n = 18)	Control vs active PDR with bevacizumab	Control vs active PDR w/o bevacizumab	Control vs inactive PDR	Active PDR with bevacizumab vs active PDR w/o bevacizumab	Inactive PDR vs active PDR w/o bevacizumab
PIGF (pg/ml)	3.7 (3.7-3.7)	24.8 (3.7-773.5)	11.5 (3.7-211.4)	11.0 (3.7-96.9)	<0.0001*	<0.0001*	<0.0001*	0.56	0.004*
	No RD (control) (n = 19)	Active PDR w/o bevacizumab (n = 25)	Active PDR with bevacizumab (n = 19)	Inactive PDR (n = 16)	Control vs active PDR with bevacizumab	Control vs active PDR w/o bevacizumab	Control vs inactive PDR	Active PDR with bevacizumab vs active PDR w/o bevacizumab	Inactive PDR vs active PDR w/o bevacizumab
VEGF (pg/ml)	24.8 (24.8-24.8)	265.9 (24.8-5853.1)	24.8 (24.8-24.8)	35.6 (24.8-735.5)	1	<0.0001*	0.0002*	<0.0001*	0.021*

*P-value < 0.05.

group were significantly higher than those in the control group ($P < 0.0001$; Figure 1 and Table 1).

Vitreous levels of VEGF in PDR patients and relationship of bevacizumab treatment to VEGF and PIGF levels

Vitreous VEGF levels were significantly increased in the active PDR group without bevacizumab (265.9 pg/ml, range: 24.8-5853; $P < 0.0001$; Figure 2 and Table 1). The vitreous VEGF levels in the inactive PDR group (which did not receive pre-operative bevacizumab) were significantly reduced (35.6 pg/ml, range: 24.8-735.5; $P = 0.021$) compared to the active PDR group without bevacizumab (Figure 2 and Table 1). Vitreous VEGF levels in the active PDR group with bevacizumab were below the limit of detection (24.8 pg/ml), as expected. In contrast, there was no significant difference in vitreous PIGF levels between active PDR patients with and without bevacizumab treatment ($P = 0.56$; Figure 2 and Table 1).

Correlation between PIGF and VEGF in the vitreous of active PDR patients without bevacizumab treatment

We sought next to determine if there was a correlation in the levels of PIGF and VEGF in the vitreous specimens. We found that there is a highly significant correlation between the levels of PIGF and VEGF in active PDR patients without bevacizumab treatment ($r = 0.83$, $P = 0.0001$; Figures 3a and b). In order to facilitate visualization of this correlation between levels of PIGF and VEGF, we constructed a graph that excluded the three highest values (3B).

Immunohistochemical analysis of VEGF receptor 1 in PDR membranes

Using immunohistochemistry, we investigated the expression and localization of the PIGF receptor, FLT1 (also known as VEGFR1), in active PDR membranes. We were specifically interested in whether FLT1 was expressed in vascular endothelial cells by immunohistochemical staining. In PDR membranes, FLT1 expression was detected in vascular endothelial cells, as indicated by corresponding staining with the endothelial cell marker CD31/PECAM-1 (Figure 4).

Discussion

The discovery of VEGF as a major regulator of diabetic retinopathy has led to the extensive use of anti-VEGF. These medications are often used in the context of PDR, especially in the presence of vitreous haemorrhage or neovascular glaucoma.¹¹ However, these anti-angiogenic

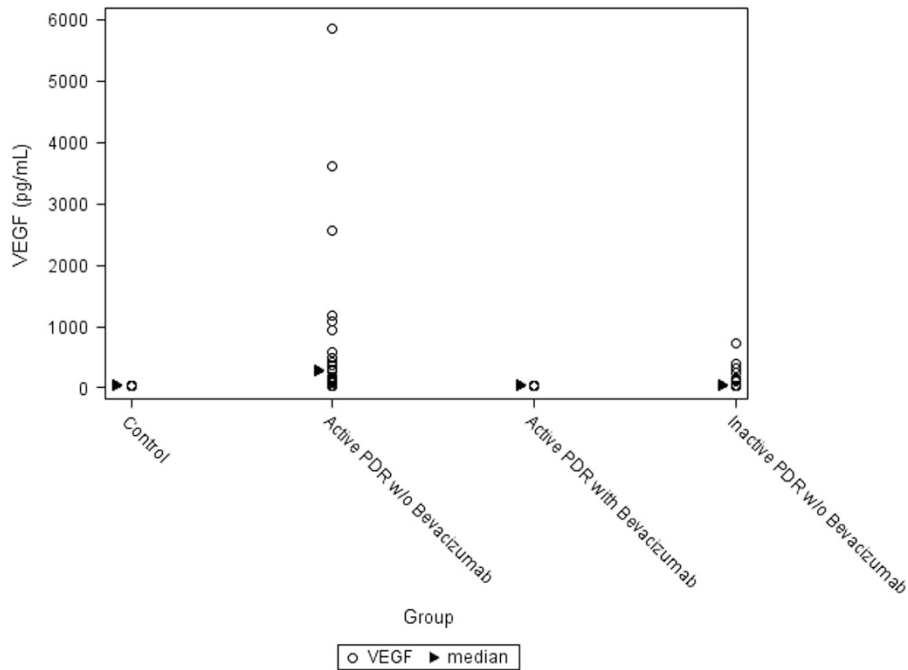


Figure 2 VEGF levels in the vitreous among active PDR without ($n = 25$) or with ($n = 19$) bevacizumab treatment, inactive PDR ($n = 16$) and no DR ($n = 19$).

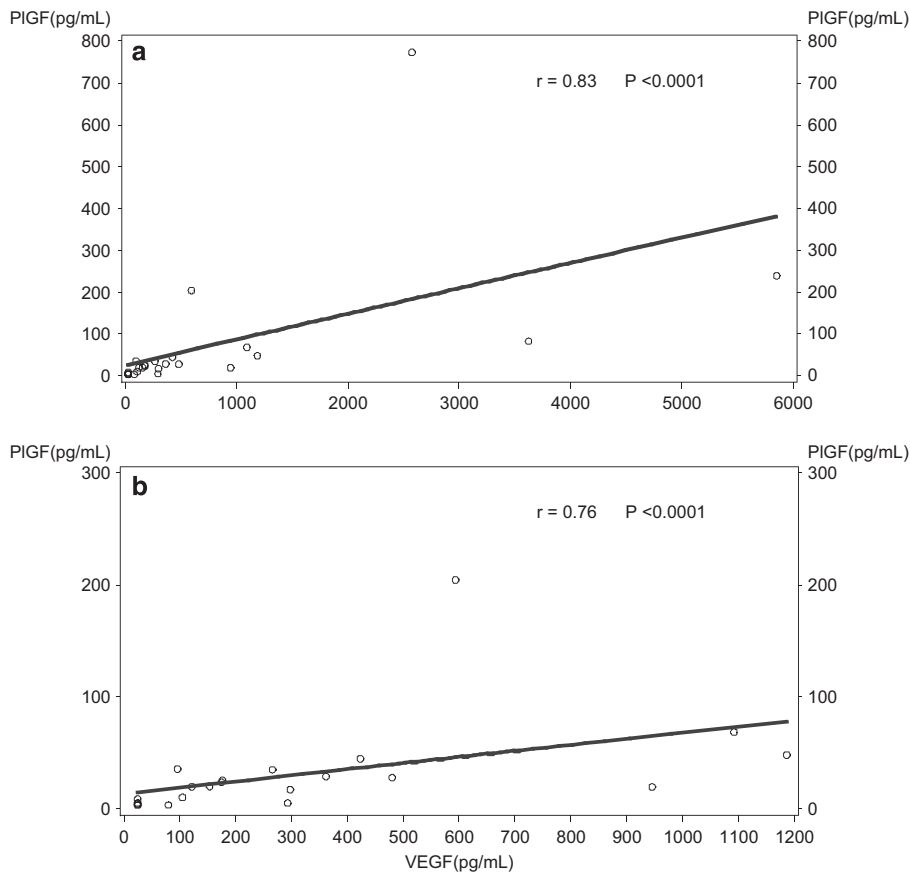


Figure 3 Correlations between vitreous levels of VEGF and PIGF in active PDR without bevacizumab treatment by Spearman's correlation coefficient test, either with inclusion of all values (a) or with exclusion of the three highest values (b). r and P values are shown in the figure.

therapies still face a number of clinical obstacles and drawbacks, such as short-lived effect and optimal dosing. There are potential safety concerns, in part based on the role of VEGF in maintenance and health of retinal cell types,¹¹ and concerns have been raised linking anti-VEGF to retinal atrophy in macular degeneration¹⁹ and to development of glaucoma.²⁰ As a result, it is of great interest to identify additional factors that may play a role in PDR and diabetic retinopathy in general.

Basic research and pre-clinical studies suggest that PIGF could be a major pathogenic molecule in diabetic retinopathy. However, relatively few human studies have been presented to support the role of PIGF in human DR.^{17,18} A very recent study demonstrated that vitreous PIGF levels in patients with neovascular glaucoma associated with ischaemic retinal diseases were even higher than those in PDR patients without NVG.²¹ This correlation of PIGF levels with severity of retinal ischaemia further suggests its pathogenic role. Our current findings that vitreous PIGF levels are reduced in quiescent PDR compared to active PDR further bolsters the likely importance of PIGF in human PDR. In addition, our immunohistochemical studies demonstrating the expression and localization of the PIGF receptor, FLT1, in vascular endothelial cells in human PDR membranes solidify the relevance of elevated PIGF levels for retinal angiogenesis. Strikingly, a previous study found that PIGF intensely localized to the endothelial and perivascular region of newly formed blood vessels of excised fibrovascular preretinal membrane in patients with PDR. However, PIGF was weak or absent in diabetic retina without neovascularization and was absent in normal retina and in diabetic retina following extensive panretinal photocoagulation.¹⁷

An additional facet of our study was the very strong correlation between vitreous levels of PIGF and VEGF in active PDR. This would be consistent with a common stimulus that upregulates expression of both growth factors. Indeed, studies in mouse models of ischemic retinopathy have implicated HIF-1 α as a transcription factor that induces both VEGF and PIGF in the context of hypoxic retinal disease.²²

The likely importance of PIGF as an additional pathogenic factor in diabetic retinopathy is interesting in light of the current extensive use of anti-VEGF agents in Saudi Arabia and worldwide and may offer novel opportunities for additional therapeutic strategies. In comparison to anti-VEGF, treatment with anti-PIGF antibody (α -PIGF) might provide therapeutic benefits without the potential side effects of anti-VEGF drugs as noted above. Interestingly, the therapeutic potential of a humanized version of α -PIGF (TB403) is being evaluated in cancer patients.²³ Conceivably, this and future anti-PIGF drugs could be considered as new treatment options

for both PDR and diabetic macular oedema with improved efficacy.

Recently, aflibercept has been demonstrated to achieve better visual outcomes than bevacizumab and ranibizumab in certain contexts, including a subgroup of patients with diabetic macular edema.²⁴ A potential differentiating property of aflibercept is its ability to bind PIGF in addition to VEGF, leading to speculation that this characteristic might render aflibercept more efficacious.^{25,26} A recent pre-clinical study has demonstrated the importance of PIGF in experimental diabetic retinopathy.¹⁰ Particularly relevant to PDR, basic research and pre-clinical studies have demonstrated that PIGF plays a role in promoting the angiogenic phenotype in endothelial cells and in stimulating angiogenesis *in vivo*, including a mouse model of ischaemia-induced retinal neovascularization.^{9,27}

Although aflibercept has a direct effect in binding PIGF, it is plausible that bevacizumab and ranibizumab, via their anti-VEGF effect, might also influence PIGF levels. VEGF has previously been shown to upregulate PIGF expression.^{15,16} Studies indicate that preoperative treatment with bevacizumab may influence vitreous levels of some cytokines and growth factors, including SDF-1¹³ and some inflammatory cytokines.¹⁴ We were therefore interested in determining whether preoperative treatment with bevacizumab influenced vitreous levels of PIGF. In the current study, we did not find evidence to suggest such an effect.

Although our study demonstrates a strong correlation of PIGF levels with PDR disease status, it does not establish a definitive cause and effect relationship between PIGF and PDR progression, and additional studies will be required. In addition, the findings relate specifically to PDR and not diabetic macular oedema due to the inherent limitations in access to vitreous specimens from the latter condition. With regard to the effect of anti-VEGF treatment, it would be helpful to include studies in the future that address the effect of aflibercept, given its known property for binding both PIGF and VEGF. Finally, we acknowledge the relatively small sample size for some of our groups, although the very low *P*-values in our study strongly support our major conclusions. Larger follow-up studies will likely be useful to solidify these findings.

In conjunction with previous studies, our findings lend further support to the concept that PIGF plays an important role in proliferative diabetic retinopathy. This raises the possibility that aflibercept may provide an added advantage over other current anti-VEGF agents. Further studies will be required to confirm the potential benefit conferred by suppression of PIGF in retinal diseases including proliferative diabetic retinopathy.

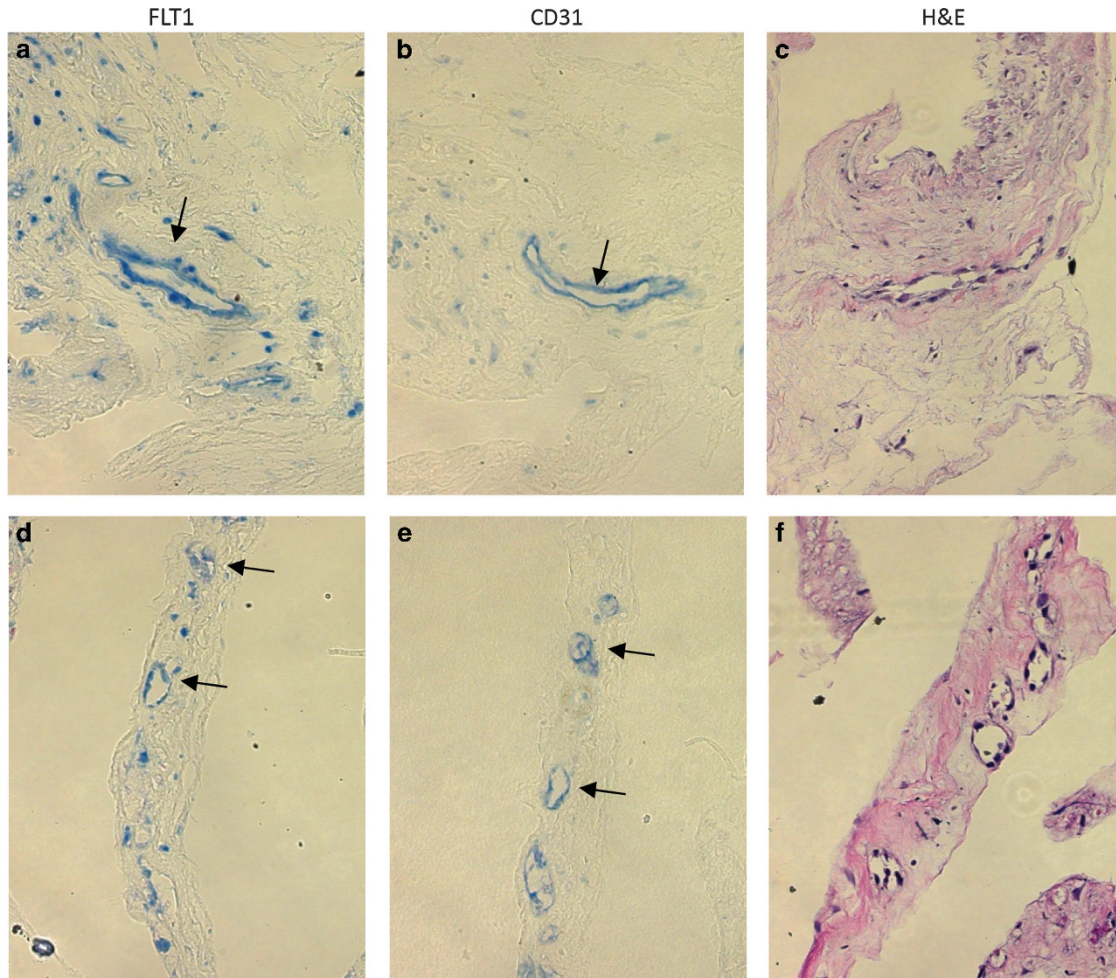


Figure 4 The expression of FLT1 in PDR membranes. (a, b) IHC staining of FLT1 in membranes from active PDR patients. FLT1 is expressed in endothelial cells lining the lumen of blood vessels (black arrows). (c, d) CD31 staining confirmed the distribution of microvascular endothelial cells in adjacent sections from PDR membranes that were used in (a) and (b). (e) H&E staining in adjacent sections from PDR membranes that were used in (a) and (b). The images were taken using $\times 20$ objective.

Summary

What was known before

- Placental growth factor (PIGF) promotes angiogenesis in cultured endothelial cells and stimulates retinal and systemic angiogenesis in mouse models.
- PIGF is increased in vitreous in active proliferative diabetic retinopathy (PDR) compared to control, non-angiogenic cases.

What this study adds

- PIGF is significantly downregulated in the vitreous in inactive/quiescent proliferative diabetic retinopathy, compared to active PDR.
- PIGF levels in the vitreous are not influenced by pre-operative treatment with the anti-VEGF drug, bevacizumab.
- PIGF levels in the vitreous correlate closely with VEGF levels in active PDR.
- The PIGF receptor, FLT1, is expressed in vascular endothelial cells in PDR membranes.

Conflict of interest

The authors declare no conflict of interest.

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