

Upregulated expression of vascular endothelial growth factor in proliferative diabetic retinopathy

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Abstract

Aims/Background—Vascular endothelial growth factor (VEGF) is a hypoxia induced angiogenic factor. Recent studies have shown that high levels of VEGF accumulate in the vitreous of patients with proliferative diabetic retinopathy (PDR). The purpose of the present study was to identify the retinal cells that upregulate VEGF expression in human PDR patients representing progressive stages of retina deterioration.

Methods—Thirteen formalin fixed and paraffin embedded enucleated eyes with PDR were used (eyes were enucleated because of being blind and painful as a result of neovascular glaucoma). Thin retina sections were hybridised in situ with a VEGF specific probe, to identify cells producing VEGF mRNA.

Results—All eyes with PDR showed upregulated expression of VEGF mRNA, specifically in the cells of the neurosensory retina. VEGF expression was upregulated in all three nuclear layers – namely, the ganglion cell layer, the inner nuclear layer, and the outer nuclear layer. However, in each patient, VEGF producing cells were mostly distributed in a different layer, or even confined to a specific region in that layer. For example, expression by the outer nuclear layer was mostly detected in detached (presumably hypoxic) regions of the retina.

Conclusions—Progression of PDR is distinguished by a sustained, upregulated expression of VEGF by the neurosensory retina. Cells in all retina layers can potentially contribute to augmented VEGF production. The restricted population of VEGF producing cells in each case is likely to represent cells residing in ischaemic regions of the retina. Thus, VEGF may function as a linking factor between retinal ischaemia and PDR associated neovascularisation.

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Intraocular neovascularisation is one of the major causes of blindness worldwide.¹ Diabetic retinopathy, and especially its proliferative stage, is the second leading cause of legal blindness, and the leading cause of blindness in people 25 to 74 years of age, in the United States.²

The retina has the highest oxygen requirements of any tissue in the body. Hence, insufficient perfusion and the resultant hypoxia are considered to be a major stimulus for

compensatory neovascularisation. Michaelson³ was the first to hypothesise that the ischaemic retina elaborates a hypoxia inducible angiogenic factor. Ashton⁴ later extended the theory by postulating that the vasoformative material produced by the ischaemic retina not only induces retinal neovascularisation, but may also diffuse anteriorly to stimulate new vessel formation in the iris.

Many investigators have searched for Michaelson's postulated angiogenic factor. In recent years, several candidate factors were suggested to play a role in proliferative diabetic retinopathy (PDR) pathogenesis. Insulin-like growth factor 1 (IGF-1) and its receptors were detected in the vitreous and retina, with elevated levels of IGF-1 detected in the vitreous of eyes with PDR.^{5,6} Fibroblast growth factor (FGF), both acidic FGF and basic FGF, were found in eyes with PDR.^{7,8} Transforming growth factor β (TGF- β), platelet derived growth factor (PDGF), and tumour necrosis factor α (TNF- α),⁹ were also suggested to play a role in ocular angiogenesis. More recently, it was shown that increased vitreous levels of vascular endothelial growth factor (VEGF) are found in PDR patients.^{10–12}

The question of which of these factors, if any, functions to link retinal ischaemia with the pathological angiogenic response in PDR patients, however, has remained unanswered. We have previously shown that VEGF is hypoxia inducible and may mediate hypoxia initiated angiogenesis, in general.¹³ These observations were later extended to show that induction of experimental retinal ischaemia in animal models leads to upregulated VEGF expression.^{14,15} These findings prompted us to examine whether VEGF expression is upregulated during natural progression of human PDR, to identify the VEGF producing cells at progressive stages of PDR development, and to determine whether VEGF is specifically induced in ischaemic regions of the retina.

Material and methods

The files of the FC Blodi Eye Pathology Laboratory were searched for paraffin blocks of enucleated eyes with the histopathological diagnosis of proliferative diabetic retinopathy and neovascular glaucoma. Cases were excluded if the eyes were phthisical or were complicated by endophthalmitis. Importantly, no postmortem specimens were included in this study. Thirteen cases described clinically as 'blind and painful' qualified for this study. As a control, five eyes with choroidal or ciliary body melanoma, but with no detectable neovascularisation, were analysed in parallel.

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Enucleated eyes were fixed in 10% neutral buffered formalin for 2–7 days. Five mm thick sections at the pupillary-optic nerve (P-O) level were collected on poly (L) lysine coated glass slides, refixed, dehydrated in graded ethanol solutions, and further processed for in situ hybridisation as follows. Sections were pre-treated successively with 0.2 M HCl, pronase (0.125 mg/ml), 4% paraformaldehyde, and acetic anhydride in triethanolamine buffer. Hybridisation was carried out at 50°C overnight in a solution containing 50% (vol/vol) formamide/0.3 M NaCl and ³⁵S-labelled RNA probe (2×10⁸ cpm/ml). A 590 bp cDNA fragment that includes most of the coding region of human VEGF₁₆₅ and subcloned in a PBS vector (Stratagene), was used as a VEGF specific probe. The linearised plasmid served as a template for synthesis of an ³⁵S-labelled complementary RNA in either the antisense or sense orientation (using T3 or T7 RNA polymerase). The cRNA was fragmented by a mild alkaline treatment to a size of 50–150 bp before use for in situ hybridisation. Post-hybridisation washing was performed under stringent conditions that included an incubation at 50°C for more than 14 hours in 50% formamide/0.3 M NaCl, and a 30 minute incubation at 37°C with RNAase (20 mg/ml). Autoradiography was performed using Kodak NTB-2 nuclear track emulsion (Kodak, Rochester, NY, USA) with exposure for 5–9 days. Slides were examined by light microscope, using bright field (to see the black grains) as well as dark field (by which the grains look bright).

Results

The clinical details of the 13 patients included in the study are summarised in Table 1. There were 10 males and three females aged 19 to 81 years, all with diabetes mellitus. There were eight right eyes and five left eyes. All were enucleated because they were blind and painful due to neovascular glaucoma. Histologically, all had proliferative diabetic retinopathy and neovascular glaucoma. Nine had partial or total retinal detachment. Eleven patients showed evidence of intraocular haemorrhage, to a certain extent. Eight patients were previously treated by laser retinal

photocoagulation. The specimens examined represented progressive stages in severity of the disease, as evident also by different degrees of retinal disorganisation (see below).

To identify cells expressing VEGF mRNA, in situ hybridisation analysis was performed, using sections of whole globes. Preliminary experiments indicated that the mRNA preservation in these specimens is adequate for detection by in situ hybridisation analysis, even after long term storage (certain specimens have been stored as paraffin blocks for longer than 5 years).

In comparison with control eyes (eyes with no intraocular neovascularisation), where VEGF mRNA was either undetectable or barely detectable, all cases of PDR showed strong in situ hybridisation signals. Control hybridisations of adjacent serial section with a VEGF specific probe in the 'sense' orientation showed no signal above background (data not shown). The retina was the only tissue in the eye in which expression of VEGF was observed. Four representative examples of retina with PDR are shown in Figure 1.

Comparative analysis between PDR patients with respect to the pattern of VEGF expression allowed us to make the following generalisations: (1) Abundant VEGF expression was detected in all three nuclear layers of the retina – namely, in the ganglion cell layer (GC), in the inner nuclear layer (INL), and in the outer nuclear layer (ONL). Strikingly, however, in each patient VEGF was predominantly produced by cells residing in one (or two) particular cell layers. Furthermore, the cellular layer producing the bulk of VEGF mRNA differed from one case of PDR to the other. For example, in patient 4 VEGF was mostly produced by cells residing in the ganglion cell layer (Fig 1A), in patient 10 VEGF expression was mostly confined to the INL (Fig 1B), and in patient 8 both INL expression and ONL expression were detected (Fig 1D). (2) In some cases, the territory of VEGF production was further restricted to certain regions within a single layer. Examples can be seen in Figure 1A, where ganglion cells in a particular region express more VEGF than ganglion cells in its flanking regions, and in Figure 1D, where a limited subset of INL cells contribute to VEGF production significantly more than neighbouring INL cells. (3) Regarding the relation between VEGF production and the status of retinal disorganisation, we note that upregulated VEGF expression is already detectable in relatively early stages of PDR progression, distinguished by the grossly normal appearance of the retina (for example, case presented in Figs 1A and 1B), continues at stages where the structural organisation of the retina is severely impaired (for example, case shown in Fig 1C) and still persist in the fully deranged retina (for example, in the 'funnel-shaped' retinal detachment shown in Fig 1D). Expression of VEGF in the ONL was only detected in cases of retinal detachment (see, for example in Fig 1D).

Table 1 Clinical features of blind painful eyes removed for proliferative diabetic retinopathy and neovascular glaucoma

Patient No	Age (years)	Sex	Eye	History	Haemorrhages	Other findings
1	77	F	R	DM – at 48 years	Subretinal	Aphakia
2	33	M	R	IDDM	Hyphaema, vitreous, subretinal	PRP
3	55	M	L	DM – at 29 years	Hyphaema, subretinal	
4	26	M	L	DM		NVD, PRP
5	24	M	L	IDDM	Massive, intraocular	PRP
6	58	M	R	DM	Hyphaema, retinal, subretinal, subhyaloid	PRP
7	42	M	R	DM	Subretinal	PRP
8	19	F	R	DM	Hyphaema, vitreous, subretinal, suprachoroid	
9	72	F	L	DM		
10	56	M	R	IDDM	Vitreous, subretinal	PRP
11	81	M	R	DM	Hyphaema, vitreous	NVD, PRP
12	41	M	R	DM	Subretinal, vitreous	
13	59	M	L	DM	Vitreous, subretinal, intraretinal	PRP

DM=diabetes mellitus (type not stated); IDDM=insulin dependent DM; PDR=proliferative diabetic retinopathy; PRP=panretinal photocoagulation (laser); NVD=neovascularisation of optic disc.

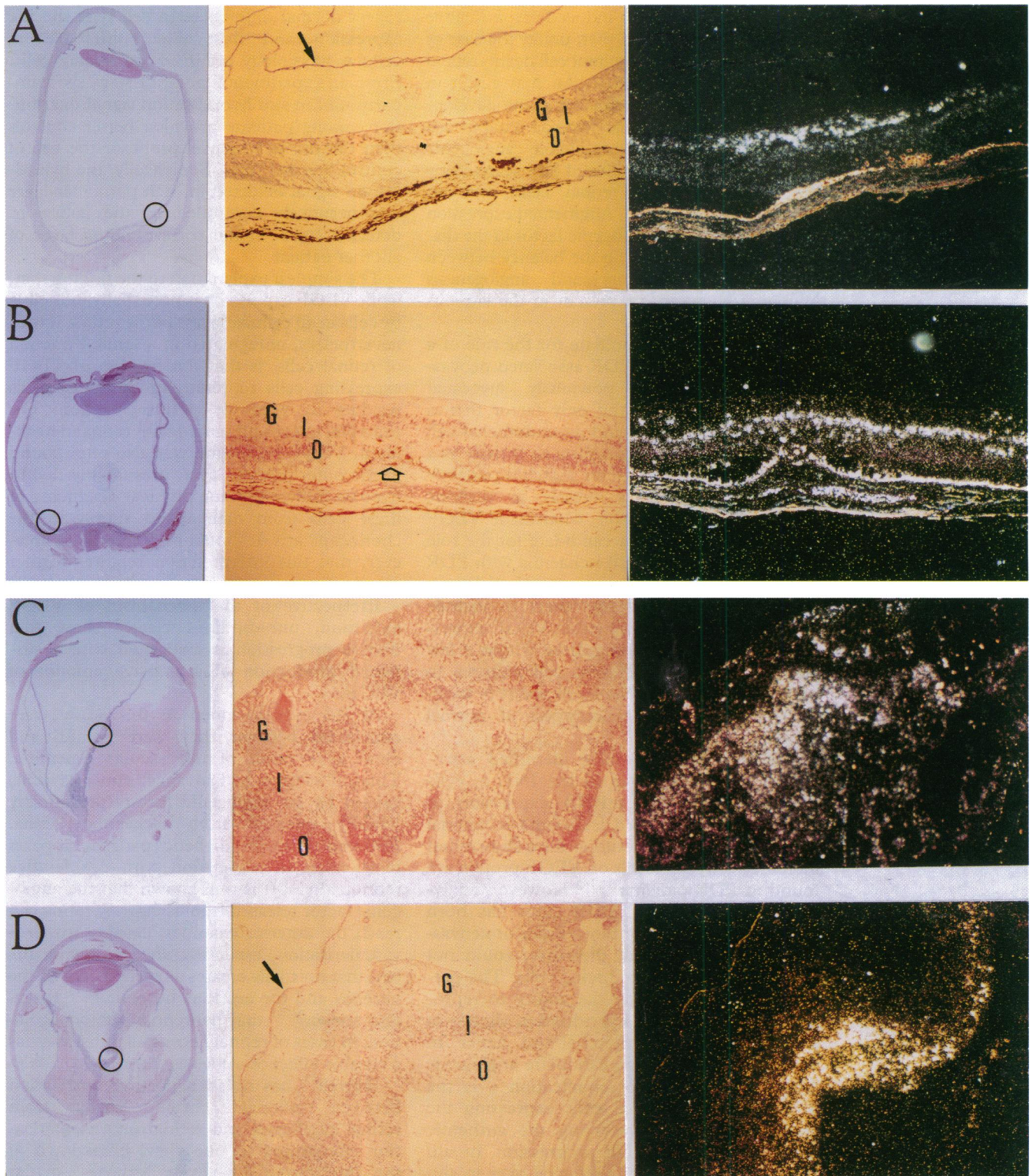


Figure 1 Expression of vascular endothelial growth factor (VEGF) mRNA in the retina of patients with proliferative diabetic retinopathy (PDR). Specimens shown are from four PDR patients representing different degrees of retinal disorganisation. Images show signals obtained from *in situ* hybridisation of whole enucleated globes with a VEGF specific probe. The cases shown in (A-D) are of patients 4, 10, 6, and 8, respectively (Table 1). Left panels show a general view of the enucleated globe, stained with haematoxylin and eosin ($\times 1.25$ magnification). Middle and right panels show (at a higher ($\times 500$) magnification) a region of the retina from an adjacent section hybridised with the VEGF specific probe (a region grossly corresponding to the encircled region in the left panels). Images were photographed under both bright field (middle images) and dark field (right images) illuminations. The white staining over the pigmented epithelium, seen in the dark field image of (A) and (B) does not reflect an autoradiographic signal, but is due to pigment staining. Arrows point at neovascular membranes. The open arrow in (B) points at an old laser hit. G=ganglion cell layer; I=inner nuclear layer. O=outer nuclear layer.

Discussion

Early vascular changes in PDR include degeneration and loss of pericytes¹⁶ and basement membrane thickening.¹⁷ Both changes are late consequences of the systemic metabolic abnormalities associated with prolonged hyperglycaemia. The early changes in the retinal vasculature may eventually lead to a

compromised blood flow and to severe ischaemia of the sensory retinal tissue. Subsequent steps in PDR pathogenesis are believed to represent a compensatory angiogenic response leading to excessive formation of abnormally leaking vessels.¹⁸

Following Michaelson's³ and Ashton's⁴ initial hypothesis that retinal neovascularisation

is triggered by a hypoxia induced vasoformative factor, Wise¹⁹ suggested that, unlike the case of retinal infarction and resultant cell death, retinal venous or capillary obstruction may result in retinal circulatory impedance and the development of a relative cellular hypoxia without cell death. Thus, hypoxia inducible angiogenic factor is likely to be produced and secreted by the affected sensory retinal cells over an extended period of time. Augmented expression of hypoxia induced angiogenic factor in the diabetic patient may then tip the balance between angiogenesis stimulators and angiogenesis inhibitors known to be present in the vitreous and retina.²⁰⁻²⁴

VEGF is a good candidate for the role of a factor responsible for PDR associated neovascularisation. Among previously suggested candidates, VEGF is the only factor that is both a secreted factor and an endothelial cell specific mitogen. Furthermore, its activity as a vascular permeability factor matches perfectly the findings of leaky vessels in diabetic retinopathy.²⁵⁻³¹ However, what made VEGF an even more attractive candidate for the role of a factor linking retinal ischaemia with PDR associated angiogenesis, were our initial findings,¹³ later extended by others to include additional tissues, showing that VEGF expression is dramatically upregulated within a few hours of exposing different cell cultures to hypoxia, and that mRNA levels revert to background levels upon resumption of normal oxygen supply.

Recent studies^{14 15} have identified astrocytes and cells residing in the INL as cells upregulating VEGF mRNA expression in experimentally induced ischaemia in animal models. Other recent studies have shown that increased vitreous levels of VEGF protein are found in PDR patients.¹⁰⁻¹² However, analysis of natural human PDR cases has been limited to analysis of vitreous fluids or neovascular membranes and, therefore, could not identify the VEGF producing cells. The present study – exploiting the ability to reproduce efficient *in situ* hybridisation signals in archival pathological material and, hence, to identify cells expressing candidate angiogenic factors at the time of enucleation – is the first to identify retinal cells expressing the angiogenic factor, preserving the authentic cellular contexts of PDR progression. *In situ* analysis of mRNA was preferred over *in situ* immunodetection of the encoded protein because the localisation of the mRNA unequivocally identifies the producer cells, whereas VEGF is known to be secreted and might also be sequestered elsewhere in the tissue.³²

If the assumption that VEGF expression in PDR is upregulated in response to retinal hypoxia is correct, then it is anticipated that in different patients, different populations of retinal cells will be the major producers of VEGF mRNA. Indeed, we found that in each patient, VEGF producing cells were mostly distributed in a different layer, or even confined to a specific region within a single layer. Clearly, these patterns of expression cannot

reflect normal differences in cell type specific expression, and must reflect differences in cellular physiology of VEGF producing cells. From an experimental point of view, the comparison of *in situ* hybridisation signal distribution among patients provided better controls than the use of a 'sense' probe or the use of control 'normal' eyes. Specifically, the hybridisation signal detected in each particular layer was negatively controlled by the failure to detect a signal in the corresponding layer of another patient.

The simplest explanation to the finding that, while VEGF expression is potentially inducible by cells in all cellular layers of the retina, it was, nevertheless, upregulated in a limited fraction of retinal cells, is that the fraction of VEGF expressing cells represent in each case cells residing in a poorly perfused region. For example, in eyes presented with retinal detachment, VEGF expression was also often found in the outer nuclear layer, presumably due to hypoxia inflicted by the physical distancing of these cells from their blood supply in the choriocapillaris. It should be pointed out, however, that this interpretation requires further proof.

Irrespective of the mechanism of VEGF induction, our findings clearly show that disease progression is associated with a sustained production of a massive amount of VEGF.

All 13 eyes included in this study were enucleated because they were painful and blind as a result of neovascular glaucoma. Thus, all of them had advanced rubeosis iridis, caused by an angiogenic factor (presumably VEGF) released from ischaemic retina. Rubeosis iridis in diabetic patients appears most often in association with proliferative retinopathy.³³ It is well known that the angiogenic factor released from ischaemic retina can reach the anterior chamber, causing iris neovascularisation, especially after removing the lens that normally creates a barrier between the vitreous and the aqueous humour.^{34 35} It was also shown^{36 37} that panretinal photocoagulation, in cases of retinal neovascular disease, has a curable effect on rubeosis iridis.

Hayreh³⁸ has noted a positive correlation between prolonged chronic leakage from retinal capillaries and retinal neovascularisation. According to one view, breakdown of the blood-retinal barrier exposes ocular tissues and vessels to 'abnormal compounds' that induce neovascularisation. Since VEGF also acts as a vascular permeability factor, it seems more likely that increased vascular leakage is not the cause of retinal neovascularisation, but rather a consequence of excessive VEGF production.

In conclusion, our findings regarding upregulated expression of VEGF mRNA in the nuclei of sensory cells residing in hypoxic microenvironments of the PDR retina, in conjunction with previous findings regarding high levels of VEGF protein in the vitreous of eyes with PDR,¹⁰⁻¹² strongly suggest that VEGF might function as one of the factors linking retinal ischaemia and angiogenesis in PDR.

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