

# Levels of vascular endothelial growth factor are elevated in the vitreous of patients with subretinal neovascularisation

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## Abstract

**Background**—Vascular endothelial growth factor (VEGF) has been shown to play a major role in intraocular neovascularisation in ischaemic retinal diseases. Subretinal neovascularisation is an important cause of central visual loss, but little is known about the role of this growth factor in its pathogenesis. The aim of this study was to investigate the possible role of VEGF in the development of subretinal neovascularisation.

**Methods**—Undiluted vitreous samples were obtained from patients undergoing vitrectomy for removal of non-age-related subfoveal neovascular membranes (SFNM). For comparison vitreous from patients undergoing vitrectomy for idiopathic full thickness macular holes (FTMH) and proliferative diabetic retinopathy (PDR) was used. Indirect enzyme linked immunosorbent assay (ELISA), with an antibody directed against the conserved N-terminal region of human VEGF<sub>165</sub>, was used to determine vitreous levels of VEGF. The growth factor was also localised in the vitreous of patients with SFNM by western blot analysis.

**Results**—The mean (SE) VEGF concentration in the vitreous of patients with SFNM was 27.78 (2.22) ng/ml (n=8), FTMH was 16.62 (0.9) ng/ml (n=18), and PDR was 37.77 (3.28) ng/ml (n=16). The differences between the PDR group and SFNM group versus the FTMH group were both significant (p=0.0001 and p=0.0015) as analysed by the Wilcoxon rank sum test).

**Conclusions**—Vitreous levels of VEGF are significantly elevated in eyes with non-age-related subretinal neovascularisation compared with eyes with FTMH but not as elevated as in PDR. This suggests that VEGF is involved in subretinal angiogenesis.

(*Br J Ophthalmol* 1996; 80: 363–366)

In 1948 Michaelson<sup>1</sup> proposed that a chemical angiogenic factor released by ischaemic retina probably causes retinal neovascularisation. Results from recent studies suggest that vascular endothelial growth factor (VEGF), also known as vascular permeability factor (VPF)<sup>2</sup> or vasculotropin,<sup>3</sup> plays a major role in the development of intraocular

neovascularisation.<sup>4,5</sup> VEGF is a secreted 46 kDa polypeptide that has four homodimeric forms as a result of alternative splicing (giving peptides of 206, 189, 165, and 121 amino acids).<sup>6</sup> It is a potent and specific mitogen for vascular endothelial cells.<sup>7</sup> The expression of VEGF is upregulated by hypoxia, transforming growth factor  $\beta$  (TGF $\beta$ ), and by intraocular inflammation.<sup>8–10</sup> Consistent with its endothelial cell mitogenicity, VEGF has also been shown to promote new capillary formation when added on the chick chorio-allantoic membrane.<sup>6</sup> Besides angiogenic activity, VEGF also displays the physiological function of increasing the permeability of capillary microvessels to different macromolecules.<sup>11</sup>

The concentration of VEGF has been shown to decrease in the eye after successful laser treatment for proliferative retinopathies and when neovascularisation becomes inactive without treatment.<sup>4</sup> Additionally, the administration of antibody directed against VEGF has been shown to inhibit neovascularisation in retinal ischaemia.<sup>12</sup> Thus, VEGF would seem to fulfil the criteria for the long sought angiogenic factor in that its concentration in the eye is elevated in ischaemia, it is diffusible into the vitreous and anterior chamber, it stimulates endothelial cell proliferation, and its concentration significantly falls with involution of active new vessels.

While there seems to be convincing evidence that VEGF has an important role in the development of preretinal, optic disc, and iris neovascularisation in ischaemic retinopathies, little is known regarding the potential role of VEGF in the pathogenesis of subretinal neovascularisation. Subfoveal neovascular membranes (SFNM) are often devastating to central vision and have generally been considered untreatable in terms of restoring central vision because laser treatment permanently destroys foveal photoreceptors. However, Thomas and Kaplan demonstrated that surgical excision and restoration of visual acuity was possible in selected cases of SFNM.<sup>13</sup> While the treatment of SFNM may be advancing, our understanding of the pathogenetic processes is still incomplete, especially with regard to the role of growth factors in subretinal angiogenesis. The purpose of this report is to describe our findings of elevated levels of VEGF in the vitreous of patients undergoing vitrectomy for removal of non-age-related subfoveal neovascularisation.

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Accepted for publication  
19 January 1996

## Materials and methods

### STUDY POPULATION

Patients referred to the retinal diagnostic department at Moorfields Eye Hospital with a diagnosis of non-age-related SFNM had a complete ophthalmic examination including best corrected Snellen visual acuity, dilated fundus examination, and stereoscopic colour fundus photography and fluorescein angiography. All patients were judged to have SFNM anterior to the retinal pigment epithelium (type 2 SFNM according to the classification of Gass<sup>14</sup>). No patients had drusen in either eye or any other evidence of age-related macular degeneration. After confirmation that the SFNM was the cause of the patient's visual loss, an extensive explanation of the relative risks and benefits of vitrectomy and removal of the SFNM was given to each patient. Those patients who elected to undergo surgery were included in the study.

The study consisted of eight patients, seven women and one man, whose mean age was 31.5 years (range 17–50 years). The underlying diagnoses consisted of idiopathic SFNM (three cases), punctate inner choroidopathy (PIC) (two cases), multifocal inner choroidopathy (MIC) (one case), quiescent sympathetic ophthalmitis (one case), and traumatic choroidal rupture (one case). The mean duration of the SFNM preoperatively was 6 months (range 3–16 months). The comparison group consisted of 18 patients with idiopathic full thickness macular hole (FTMH) and of 16 patients with advanced proliferative diabetic retinopathy (PDR) and traction retinal detachment involving the macula, all of whom underwent vitrectomy. The group of patients with macular holes consisted of 13 women and five men with a mean age of 62 years (range 24–77) and with no evidence of age-related macular degeneration in either eye. Of the diabetic patients, seven were women and nine were men and their mean age was 42 years (range 33–65).

### VITREOUS SAMPLES

All study and comparison cases had vitreous samples obtained intraoperatively in the same manner. A standard three port pars plana vitrectomy was performed in all cases. Before turning on the intravitreal infusion, an undiluted sample (0.5–1.0 ml) of vitreous was obtained by aspiration into a 2 ml syringe attached to the vitreous cutter. The syringe with the sample was then frozen at  $-70^{\circ}\text{C}$  until it was required for analysis. The operation was then completed in the usual fashion.

## Laboratory methods

### ELISA FOR VEGF

Enzyme linked immunosorbent assay (ELISA) of undiluted vitreous samples and human recombinant VEGF<sub>165</sub> (R & D Systems, Abingdon) was carried out to quantitate the levels of VEGF. The samples were dissolved in

bicarbonate buffer, pH 9.6, and then coated on 96 well microtitre plates (ICN Biomedicals, Thame) and incubated overnight at room temperature. The wells were then washed with 20 mM TRIS pH 7.5, 0.05 M NaCl, and 0.05% Tween-20 (TRIS buffered saline, TBST) and blocked by incubation for 1 hour at  $37^{\circ}\text{C}$  with TBST containing 3% bovine serum albumin (BSA) (Sigma, Poole) and 1:50 normal goat serum (NGS) (Sigma). After washing, polyclonal anti-VEGF<sub>165</sub> antibody (kind gift of Dr H Weich, Department of Gene Expression, Braunschweig, Germany) diluted 1:1000 in TBST was added and the plates incubated for 1 hour at  $37^{\circ}\text{C}$ . After washing biotinylated anti-rabbit IgG (final dilution 1:20 000 in TBST containing 1% BSA) was added. After 1 hour of incubation at  $37^{\circ}\text{C}$  the wells were washed and extravidin alkaline phosphatase (final dilution 1:20 000 in TBST containing 1% BSA) added. The reaction was developed utilising *p*-nitrophenyl phosphate (pNPP, Sigma) as a colorimetric substrate, and the absorbance was measured at 405 nm after 1 hour, using an automated plate reader (Novo Biolabs, Nottingham).

### WESTERN BLOT ANALYSIS

Once thawed the vitreous samples were denatured in sample buffer containing 62 mM TRIS-HCl, pH 6.8; 10% v/v glycerol; 2% sodium dodecyl sulphate (SDS); 5% mercaptoethanol; 0.001% w/v bromophenol blue). Samples were heated at  $95^{\circ}\text{C}$  for 4 minutes and then subjected to SDS polyacrylamide gel electrophoresis (SDS-PAGE) using a 10% gel. Proteins were transferred to Hybond-ECL membrane (Amersham, Aylesbury) and the presence of VEGF detected by immunoblotting. The membrane was blocked with 5% BSA in TBST. All antibody incubations were performed in 1% BSA in TBST. Antibody for VEGF<sub>165</sub> was used at a 1:1000 dilution. Primary antibody-antigen complex was detected with anti-rabbit IgG horseradish peroxidase conjugate (1:10 000) with ECL western blotting analysis system (Amersham).

### PROTEIN MEASUREMENT

Total protein was determined by using the bicinchoninic acid method (Sigma, Dorset).

### STATISTICAL ANALYSIS

The levels of VEGF in the different groups were compared by the Wilcoxon rank sum test.

Table 1 Precision of the ELISA for vascular endothelial growth factor (VEGF)

Sample No	Within assay (n=5)		Between run (n=5)	
	Mean (SD)*	CV (%)	Mean (SD)*	CV (%)
1	1.127 (0.0063)	5.88	1.0279 (0.0064)	6.2
2	2.536 (0.0131)	5.20	2.5944 (0.0124)	4.8
3	5.354 (0.0402)	7.51	5.1288 (0.0333)	6.5

\*ng/ml of VEGF. CV=coefficient of variation.

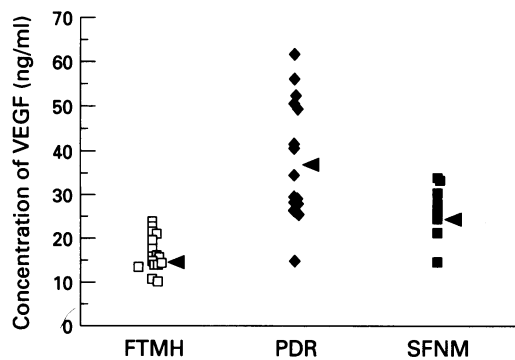


Figure 1 Vascular endothelial growth factor (VEGF) concentration in vitreous of patients undergoing vitrectomy for removal of non-age-related subfoveal neovascular membranes (SFNM), idiopathic full thickness macular holes (FTMH), and proliferative diabetic retinopathy (PDR). The mean concentrations of VEGF (arrowhead) in vitreous from SFNM, PDR, and FTMH groups were 27.8 (2.22), 37.77 (3.28), and 16.62 (0.9) ng/ml, respectively.

### Results

The level of VEGF in the vitreous was detected with a specific antibody using ELISA with sensitivity of 0.5 ng/ml. The intra-assay variation for this ELISA was 5.9–7.5% and the interassay variation was 4.8–6.5% (Table 1). The mean level (SE) of VEGF in the SFNM group was 27.78 (2.22) ng/ml (n=8) and in the FTMH group was 16.62 (0.9) ng/ml (n=18). The difference between these two groups was significant ( $p=0.001$ , Wilcoxon rank sum test).

The levels of VEGF were highest in eyes with PDR with a mean level of 37.77 (3.28) ng/ml (n=16). These levels were significantly higher than in FTMH group ( $p=0.0001$ ) and the SFNM group ( $p=0.028$ ). The difference between all three groups is illustrated graphically in Figure 1. The presence of VEGF was also confirmed by western blot analysis. Vitreous samples denatured in sample buffer were separated by SDS-PAGE on 10% gel. An immunoreactive band corresponding to molecular weight of 42 kDa was detected in the SFNM and FTMH samples (Fig 2). In most but not all the patients, the levels of VEGF

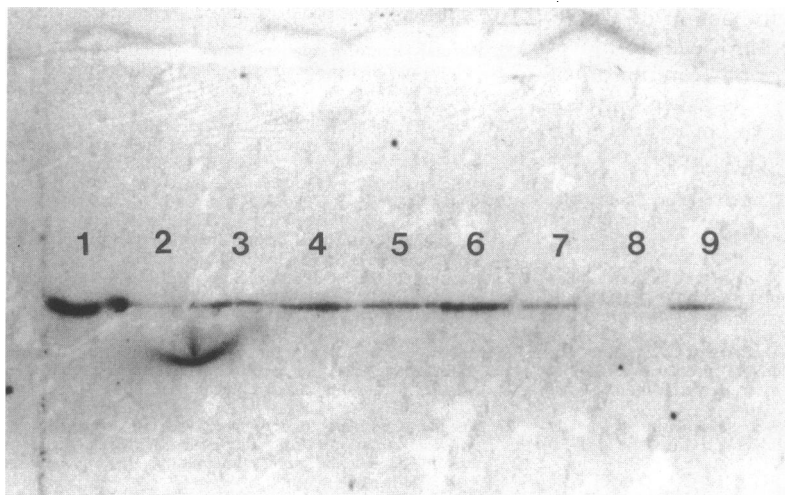


Figure 2 Western blot analysis using specific VEGF<sub>165</sub> antibody of vitreous from SFNM (lanes 2–5), FTMH group (lanes 6–9), and 2 ng of human recombinant VEGF<sub>165</sub> (lane 1): 12.5  $\mu$ l (approximately 1.5  $\mu$ g of protein) of vitreous was used for the experiment. The proteins were separated by SDS-PAGE run at 4% for the stacking gel and 10% for the running gel and then transferred to Hybond-ECL membrane.

determined by ELISA, compared well with those determined by western blot analysis and scanning densitometry (data not shown). This was perhaps expected since we lacked an internal control to make sure that an equal amount of protein from the vitreous sample was loaded per well. For this reason the western blot analysis was used mainly for qualitative and not quantitative determination of vitreous levels of VEGF.

### Discussion

Subretinal neovascularisation is a devastating complication of macular diseases, the most common being age-related macular degeneration (ARMD). Most theories of the subretinal neovascular process implicate the occurrence of breaks in Bruch's membrane as the initial step. It is likely that once these breaks occur a reparative process, similar to wound healing, is initiated that stimulates endothelial cell proliferation and fibroglial scar formation.<sup>15 16</sup>

In this study we demonstrated by ELISA and western blot analysis that VEGF is present in the vitreous of patients with subfoveal neovascular membranes (SFNM) and the levels are significantly higher compared with eyes with FTMH, which is a non-ischaemic, non-neovascular disorder. This finding differs from the recent study of Aiello *et al*<sup>4</sup> in which it was suggested that vitreous samples from patients with neovascular membrane had no detectable level of VEGF. However, some recent studies do suggest the involvement of VEGF in the pathogenesis of subretinal neovascularisation. Malecaze *et al*<sup>5</sup> have been able to demonstrate, using the method of reverse transcription-polymerase chain reaction, the VEGF is expressed in samples of neovascular membranes. Immunohistochemical studies have also localised VEGF in the retinal pigment epithelial cells (RPE) and other cells surrounding the subretinal membrane in postmortem eyes and in surgically removed membranes.<sup>17 18</sup> Although, RPE cells have been shown to synthesise and secrete VEGF in vitro and in situ<sup>19</sup> other cellular components of the neovascular membranes such as fibroblasts or macrophages could also be a likely source of VEGF. The latter are present in the majority of active subretinal neovascular complexes and represent an inflammatory component of the disease process.<sup>15 16</sup> In a recent report, Motokura *et al*<sup>20</sup> were able to demonstrate that subretinal neovascularisation can be induced in the rat by injection of VEGF into the subretinal space.

All our patients with SFNM were young with focal abnormalities of the RPE-Bruch's membrane-choriocapillaris complex due to inflammatory or idiopathic causes. A recognised stimulus of VEGF production is inflammation,<sup>10</sup> and three of the patients in the present study had recognisable evidence of prior choroiditis, in the form of inactive chorioretinal scars. Interestingly, two of these cases had the highest measurable levels of VEGF in the study group. Not surprisingly, the levels of VEGF found in cases with SFNM were significantly lower than those found in the diabetics

with proliferative diabetic retinopathy. This could be explained by the fact that SFNM is a focal process whereas PDR involves very extensive areas of retinal ischaemia. The observation that VEGF is elevated in the vitreous of patients with PDR is consistent with other reports<sup>4 5 21 22</sup> but the mean concentration of VEGF is different; 37.77 ng/ml in the present study compared with 4 ng/ml reported by Aiello *et al.*<sup>4</sup> and 1.164 ng/ml reported by Adamis *et al.*<sup>23</sup> The mean value in the present study is in line with 28 ng/ml reported by Plouet *et al.*<sup>24</sup> but is lower than 88 ng/ml reported by Malecaze *et al.*<sup>5</sup> One possibility for this large variation in VEGF levels found in the different laboratories is perhaps the timing of vitreous sampling. Aqueous levels of VEGF have been reported to decrease before regression of new vessels in monkeys with iris neovascularisation. This could also account for the large variability in the vitreous VEGF levels in patients with PDR found by us and all the other workers.<sup>4 5 23 24</sup> Variations in the standard diluent, incubation time, temperature, pipetting and age of the antibody are also likely to effect the sensitivity and the measurement of VEGF by ELISA in the different laboratories.<sup>4 5 23 24</sup>

Although photocoagulation is effective in preserving central vision in extrafoveal or juxtafoveal lesions<sup>21 25-27</sup> recurrences are common and frequently involve the fovea, resulting in permanent loss of vision.<sup>22</sup> Photocoagulation treatment of subfoveal lesions is, in general, unsatisfactory with initial profound decrease in central vision.<sup>28</sup> Subfoveal membranes can be removed by surgical techniques and restore acuity in selected cases.<sup>13</sup> Patients with ARMD tend to fare poorly with surgery, and the best candidates seem to be those with ocular histoplasmosis or idiopathic lesions that have relatively focal areas of disease.<sup>14</sup> The findings that elevated levels of VEGF are present in the vitreous of eyes with non-age-related subretinal neovascularisation would suggest that VEGF may be similarly involved in subretinal angiogenesis. If these results are confirmed by larger studies, further investigations on the action of VEGF in the subretinal space may lead to novel therapies with antibodies or other inhibitors of VEGF that might involve less tissue destruction and better preservation of vision than photocoagulation or surgical removal.

Presented in part at the 1995 ARVO meeting, Fort Lauderdale, FL, USA.

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