LABORATORY SCIENCE

Plasma vascular endothelial growth factor, soluble VEGF receptor FLT-1, and von Willebrand factor in glaucoma

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Aim: To investigate plasma indices of vascular permeability (vascular endothelial growth factor, VEGF—also an index of angiogenesis, as well as the soluble receptor for VEGF, sFlt-1) and endothelial damage/dysfunction (von Willebrand factor, vWf) in glaucoma.

Methods: Citrated plasma was assayed for VEGF, sFlt-1, and vWf (all ELISA) in a cross sectional study of 50 patients (20 male; mean age 63.9 years, SD 10.5) with glaucoma: 26 had normal tension glaucoma (NTG) and 24 had primary open angle glaucoma (POAG), who were compared with 26 healthy controls (mean age 73.4 years, SD 9.2).

Results: Median (interquartile range, IQR) levels of VEGF were significantly elevated in patients with NTG and POAG compared to healthy controls (Kruskal-Wallis test, $p < 0.001$). Similarly, mean (SD) vWf levels were abnormal in NTG and POAG compared to healthy controls (one way ANOVA, $p < 0.001$). Median levels of sFlt-1 were significantly lower in patients with NTG and POAG, when compared to healthy controls (Kruskal-Wallis test, $p < 0.001$; $p < 0.05$ with Tukey's post hoc test for controls v POAG). There were no significant differences in VEGF, sFlt-1 or vWf levels between the NTG and POAG groups (Tukey's test, all $p = \text{NS}$). In both NTG and POAG groups, there was a significant correlation between VEGF and sFlt-1 (Spearman, NTG: $r = 0.6517$, $p = 0.001$; POAG: $r = 0.6017$, $p = 0.008$). There were no significant correlations between VEGF and sFlt-1, or with vWf among the controls.

Conclusions: The pathogenesis of optic nerve damage in both NTG and POAG may be associated with abnormal vascular permeability and endothelial damage/dysfunction, as indicated by abnormal plasma VEGF and vWf levels in these patients.

Raised intraocular pressure is the most important risk factor for the development and progression of primary open angled glaucoma (POAG). However, normal tension glaucoma (NTG) is a type of glaucoma that is not associated with raised intraocular pressure but still leads to the same progressive optic nerve dysfunction and visual field loss. The pathogenesis of this complex disorder remains poorly understood. In the absence of an elevated intraocular pressure, "vascular factors" have been postulated to play a part in the pathogenesis of NTG.^{1,2}

These "vascular factors" could be organic, vasospastic,³ or combinations of these. Risk factors that have been investigated in NTG include large vessel disease,⁴ hypertension,⁵ hypotension and shock,⁶ diabetes mellitus,⁷ increased blood lipids,⁸ abnormal blood coagulation,^{9–11} anticardiolipin antibodies and other haemostatic factors, including von Willebrand factor,¹² perfusion pressure in ophthalmic artery,¹³ and peripheral vascular endothelial dysfunction, as assessed by forearm blood flow responses to intra-arterial infusions of vasoactive agents.³ However, none of these factors separately has been established to have a strong correlation with NTG.

Vascular endothelial growth factor (VEGF) is a growth factor that can be obtained from normal and neoplastic tissues.^{14,15} Also known as vascular permeability, factor/vasodilator, of potency 50 000 times of histamine, is a 45 kDa glycoprotein secreted by tumour cells, smooth muscle cells, macrophages, and epithelial cells. Raised levels of VEGF are common in metastatic cancer and have also been reported in atherosclerotic vascular disease.¹⁴ Raised levels of VEGF in cancers are said to reflect increased angiogenesis and it may also have a role in the normal physiology of repair.^{14–16} In addition, it has been suggested that VEGF is implicated in the pathogenesis of diabetic retinopathy.^{17,18}

VEGF interacts with endothelial cells via two high affinity membrane spanning receptors, Flt-1 and KDR. The role of receptors in embryonic vasculogenesis and adult angiogenesis and its association with several diseases has been clearly established,¹⁹ and the presence of this receptor has also been identified on retinal endothelial cells and pericytes. A soluble form of Flt-1 (sFlt-1) has been identified in conditioned culture media of human umbilical vein endothelial cells, although the pathophysiological significance of this is uncertain.²⁰

We hypothesised that abnormal VEGF levels in NTG may reflect abnormalities in the retinal vasculature, as evident (for example) in diabetic retinopathy.^{17,18} We investigated this further by undertaking a cross sectional pilot study of plasma indices of vascular permeability/angiogenesis (VEGF, vascular endothelial growth factor), soluble VEGF receptor Flt-1 (sFlt-1) and endothelial damage/dysfunction (vWf) in patients with NTG, which will be compared to levels in patients with POAG and healthy controls.

PATIENTS AND METHODS

We undertook a cross sectional study of patients with glaucoma and healthy controls. Patients with an established diagnosis of NTG and POAG were recruited from specialist glaucoma clinics at the Birmingham and Midland Eye Centre and Birmingham Heartlands Hospital. NTG was diagnosed using the following criteria: intraocular pressures < 21 mm Hg on phasing, open angles on gonioscopy, pathological disc cupping, visual field defect and a normal intracranial neuroimaging and normal blood tests to exclude anaemic and syphilitic optic neuropathy. POAG was diagnosed using the following criteria: pathological cupping of the optic nerve head associated with raised intraocular pressure and characteristic

Table 1 VEGF, soluble VEGF receptor Flt-1, and vWf in glaucoma compared to controls

	NTG	POAG	Controls	p Value
Number	26	24	26	
Age (years)	72.9 (10.2)	75.1 (11.0)	73.4 (9.2)	0.744
Median duration of known glaucoma (years)	1.5	3.5	...	0.04 (Mann-Whitney test)
Past medical history (n)				
Hypertension (treated)	7	13	...	
Ischaemic heart disease	5	5	...	
Cerebrovascular disease	2	3	...	
Body mass index (kg/m ²)	25.5 (3.8)	24.7 (4.5)	...	0.53 (t-test)
Current smokers (n)	3	1	3	
Receiving treatment for glaucoma (n)	14	20	...	
Systolic BP (mm Hg)	148.2 (21.6)	150.5 (24.7)	144.3 (28.0)	0.694
Diastolic BP (mm Hg)	84 (7.3)	86.7 (13.8)	79.8 (7.4)	0.051
% with blood pressure >160/90 mm Hg	31%	41%	...	
VEGF (pg/ml)	225* (110–500)	150* (118–235)	83 (13–125)	<0.001
sFlt-1 (ng/ml)	17 (6–60)	6* (2–19)	28 (18–39)	0.003
von Willebrand factor (IU/dl)	180 (53)*	189 (80)*	91 (19)	<0.001

Values are mean (SD), except for VEGF and sFlt-1, expressed as median (IQR)

NTG = normal tension glaucoma; POAG = primary open angle glaucoma; BP = blood pressure; VEGF = vascular endothelial growth factor; sFlt-1 = soluble VEGF receptor Flt-1.

*p<0.05 v controls, by Tukey's post hoc test (after logarithmic transformation for VEGF and sFlt-1).

visual field loss. Following informed consent, the patients attended the glaucoma research clinic for completion of a standard proforma including demography and measurement of blood pressure using a conventional mercury sphygmomanometer. Exclusion criteria were patients with confirmed diabetes, underlying neoplasm, or connective tissue, inflammatory or infective disorders, as well as those taking antithrombotic therapy or hormone replacement therapy. A 20 ml blood samples was taken from an antecubital vein with minimal haemostasis. Samples are anticoagulated and centrifuged at 3000 rpm and 4°C for 20 minutes. The platelet free plasma was immediately separated and frozen at –80°C.

Blood indices in patients with glaucoma were compared with healthy controls, which comprised staff members and patients admitted for minor surgical procedures (such as hernia repair, excision of sebaceous cysts, etc). The subjects were non-smokers with no clinical evidence of vascular, metabolic, neoplastic, or inflammatory disease, by careful history, examination, and routine laboratory tests. These subjects were normotensive and in sinus rhythm, and were not taking aspirin, warfarin, lipid lowering or antihypertensive drugs, non-steroidal anti-inflammatory drugs, or antibiotics.

Laboratory

Citrated plasma VEGF and sFlt-1 were measured by ELISAs using commercially available reagents and recombinant standards (R&D Systems, Abingdon, UK), as previously described.²⁸ The VEGF assay has a minimum sensitivity of 10 pg/ml, with an intra-assay coefficient of variation (CV) of 4.9% (n=18) and an interassay CV of 6.4% (n=40) at 1.6 pg/ml. The sFlt-1 assay has a minimum sensitivity of 50 pg/ml, an intra-assay CV of 3.7% (n=12) and an interassay CV of 8.8% (n=22) at 10 ng/ml. Levels of vWf was measured using an established ELISA technique using commercial polyclonal antisera (Dako, High Wycombe, UK). Intra-assay and interassay coefficients of variation for assays are <5% and <10% respectively.

Power calculation

We hypothesised that there would be a graded increase in levels of vWf among healthy controls, POAG, and NTG. To be precise, that the level POAG would be two thirds of a standard deviation higher than in the healthy controls, and that levels would be a further two thirds of one standard deviation higher in the patients with NTG. In order to achieve this we needed good data from 18 subjects per group to provide power of 0.80 for a difference of p<0.01 by analysis of variance. However, as we intended to measure two other variables (VEGF, sFlt-1) we

increased our numbers accordingly. We proposed to recruit 25 patients in each of the three groups.

Statistical methods

Data are expressed as mean (SD) or (for VEGF and sFlt-1) as median (IQR, interquartile range). Comparisons between cases and controls performed using the χ^2 test, unpaired *t* test or Mann-Whitney test, one way ANOVA or Kruskal-Wallis test as appropriate. Intergroup comparisons were undertaken using Tukey's posthoc test, after log transformation for non-parametrically distributed variables. The interactions between high blood pressure (>160/90 mm Hg) and subgroups of glaucoma (NTG, POAG) were explored using ANOVA for unbalanced data (general linear model analysis). Correlations between various markers were performed using Spearman rank correlation. Relations between clinical variables (age, sex, blood pressure, hypertension, vascular disease (that is, ischaemic heart disease or cerebrovascular disease, by conventional clinical definitions), etc) and various markers were explored using stepwise multiple regression analysis. A probability of p<0.05 was considered as statistically significant.

RESULTS

We studied 50 patients (20 male; mean age 63.9 years (SD 10.5)) with glaucoma: 26 had NTG and 24 had POAG, who were compared with 26 healthy controls (mean age 73.4 years, (9.2)).

Cross sectional analyses

Median levels of VEGF were significantly elevated in patients with NTG and intermediate in POAG, when compared to healthy controls (Kruskal-Wallis test, p<0.001; p<0.05 with Tukey's posthoc test for controls v NTG and POAG). Similarly, mean vWf levels were elevated in NTG and POAG, compared to controls (180 IU/dl, 189 IU/dl, and 91 IU/dl respectively; one way ANOVA, p<0.001; p<0.05 with Tukey's posthoc test for controls v NTG and POAG) (Table 1).

Median levels of sFlt-1 were significantly lower in patients with NTG and POAG, when compared to healthy controls (Kruskal-Wallis test, p<0.001; p<0.05 with Tukey's posthoc test for controls v POAG). There were no significant differences in VEGF, sFlt-1, and vWf levels between the NTG and POAG groups (Tukey's test, p=NS).

Subgroup analyses

Of the whole cohort, 18 patients (36%) had blood pressures of >160/90 mm Hg. When these patients were compared with

Table 2 Spearman rank correlations among VEGF, sFlt-1, and vWf in patients with glaucoma compared to controls

	Versus	NTG	POAG	Healthy controls
VEGF	sFlt-1	0.6517 p=0.001	0.6017 p=0.008	0.3009 0.135
	vWf	-0.1096 p=0.610	0.1206 p=0.633	-0.0133 p=0.949
vWf	sFlt-1	-0.301 p=0.144	-0.1586 p=0.492	0.26 p=0.20

normotensive patients, there were no significant differences in median VEGF (Mann-Whitney test, 257 v 150 pg/ml, $p=0.09$) and sFlt-1 (16 v 15 ng/ml, $p=0.61$), or mean vWf (unpaired t test, 161 v 179 IU/dl, $p=0.356$) levels. When the interactions between high blood pressure ($>160/90$ mm Hg) and subgroups of glaucoma (NTG, POAG) were explored using ANOVA for unbalanced data (general linear model analysis), there were no significant effects on median VEGF and sFlt-1, or mean vWf levels (all $p=NS$, data not shown). Being on treatment for glaucoma also did not influence levels of the research indices, nor did a previous history of vascular disease (all $p=NS$, data not shown).

Correlations and multivariate analysis

In both NTG and POAG groups, there was a significant correlation between VEGF and sFlt-1 (Spearman, NTG: $r=0.6517$, $p=0.001$; POAG: $r=0.6017$, $p=0.008$) (Table 2). There were no significant correlations between VEGF and sFlt-1 with vWf among the controls. There were no significant correlations among VEGF, sFlt-1, or vWf with age, sex, systolic blood pressure, or diastolic blood pressure in the NTG, POAG, and control groups (Spearman, all $p=NS$).

Using stepwise multiple regression analyses, there were no significant associations between VEGF or sFlt-1 levels and sex, blood pressure levels or the proportion who were hypertensive (systolic blood pressure >160 mm Hg plus or minus diastolic blood pressure >90 mm Hg) or had associated vascular disease (ischaemic heart disease or cerebrovascular disease) (data not shown). Age was the only significant determinant of vWf levels ($R^2=12.8$, $p<0.05$).

DISCUSSION

This study is limited by its cross sectional nature and the relatively small numbers of patients studied, but is adequately powered ($>80\%$) to show differences between cases and controls. While plasma levels of various indices have been related to intraocular pathology,^{21–23} there is also possibility that the abnormal plasma VEGF, sFlt-1, and von Willebrand factor do not necessarily imply its involvement in glaucoma; for example, these factors may also be related to underlying (manifest or silent) vascular disease—although no significant relation was found on subgroup analyses or stepwise multiple regression analysis, in the present study. Importantly, this study cannot determine causality, as whether the changes precede or are a consequence of glaucoma is uncertain. This can only be answered in a prospective longitudinal study of a large cohort of patients at risk of developing glaucoma, to ascertain whether various parameters at baseline are predictive. Although being treated for glaucoma made no difference to the levels of measured indices, we have not attempted to distinguish the effects of individual treatments, as the precise effects of treatment for glaucoma on these indices would need to be determined in a prospective study in untreated patients.

Nevertheless, the present study delivers an association among VEGF, sFlt-1, and vWf, and glaucoma, and is in keeping with the hypothesis that NTG development may have a relation to “vascular factors.”

Abnormal blood viscosity and peripheral vascular endothelial dysfunction have previously been related to eye disease. For example, abnormal haemorheology and endothelial dysfunction have been associated with microvascular disturbance in patients with diabetes mellitus complicated by proliferative retinopathy²⁴ and in retinal vascular occlusions.^{21–25} In a similar way, increased abnormal haemorheology and endothelial damage/dysfunction might have a role in the pathogenesis of optic nerve damage in NTG. vWf is an established marker of endothelial cell damage, which is affected in many vascular disorders and has prognostic implications.²⁶ Increased levels of vWf are secreted by proliferating endothelial cells relative to quiescent cells in vitro, and it has been suggested that raised levels in vivo, in some variants of inflammatory vascular disease may reflect increased production from new, proliferating microvessels in the artery wall.^{27–28} It is also of note that growth factors, such as vascular endothelial growth factor (VEGF), can induce the release of vWf from endothelial cells in vitro.²⁹

In the eye, numerous types of retinal cells are recognised to produce VEGF, including retinal pigment epithelial cells, pericytes, endothelial cells, Muller cells, and astrocytes.¹⁸ Intraocular VEGF levels have also been studied in animal models and human vitreous fluid, where the levels are found to be high in patients with active intraocular neovascularisation, such as proliferative diabetic retinopathy, ischaemic central retinal vein occlusion, rubeosis iridis, and retinopathy of prematurity.¹⁸ Changes in intraocular VEGF levels have also been related to effective laser treatment.¹⁸ We recently reported a pilot study of patients with proliferative retinopathy, who had significantly raised plasma levels of VEGF and vWf when compared to diabetics with background retinopathy only and healthy controls.²² Following panretinal laser photocoagulation of the patients with proliferative retinopathy, there was a significant reduction in plasma VEGF levels at 4 months follow up but no significant changes in plasma sFlt-1 or vWf levels; importantly, patients with complete resolution had a trend towards lower median VEGF levels.²² Furthermore, additional work from our group has demonstrated persisting abnormalities in haemorheological factors and vWf in retinal vascular occlusion,²¹ as well as abnormal levels of plasma VEGF and haemorheological markers in patients with age related macular degeneration.²³

In the present study, median levels of sFlt-1 were significantly lower in patients with NTG and POAG, when compared to healthy controls—as we previously reported in vascular disease.³⁰ The amount of sFlt-1 (measured as mass or mol) greatly exceeds that of VEGF, and the ELISA measures not simply the presence of sFlt-1, but that sFlt-1 which binds to VEGF (that is, a pseudo-bioassay), so that our finding may have biological relevance if, as seems possible, one function of sFlt-1 is to regulate plasma VEGF levels.^{30–32} Consequently, this excess of functional (VEGF binding) sFlt-1 over VEGF may frustrate the proposed use of VEGF as a potential therapeutic agent, as any exogenously administered VEGF would need to neutralise any circulating sFlt-1 before achieving therapeutic levels at target receptors/organs. The further pathophysiological consequences of sFlt-1 are unknown in glaucoma and, clearly, the present (novel) findings in this hypothesis testing pilot study will require further detailed mechanistic/interventional studies in larger groups of patients.

In conclusion, this pilot study suggests that the pathogenesis of optic nerve damage in both NTG and POAG may be associated with abnormal vascular permeability and endothelial damage/dysfunction, as reflected by abnormal VEGF and vWf levels respectively in these patients.

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