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Risk evaluation of outcome of vitreous surgery based on vitreous levels of cytokines

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CLINICAL STUDY

Abstract

Aim To ascertain whether vitreous and plasma levels of vascular endothelial growth factor (VEGF), interleukin-6 (IL-6) and fundus findings could predict the outcome of vitreous surgery in patients with proliferative diabetic retinopathy (PDR).

Methods Vitreous fluid samples were obtained during vitreoretinal surgery from 73 consecutive eyes with PDR. The levels of VEGF and IL-6 in vitreous fluid and plasma were determined by enzyme-linked immunosorbent assay. Patients were prospectively followed for 6 months and the postoperative outcome was analysed by logistic regression analysis.

Results No improvement and/or progression of PDR occurred in 23 (32%) of the 73 eyes (progression group). The vitreous levels of VEGF and IL-6 were significantly higher in eyes from the progression group than in eyes with regression of PDR (regression group) ($P = 0.0032$ and 0.0088, respectively). Multivariate logistic regression analysis showed that higher vitreous levels of VEGF were associated with the progression of PDR after vitreous surgery (odds ratio 2.72, $P = 0.0003$).

Conclusions High vitreous levels of VEGF identified as a significant risk factor for the outcome of vitreous surgery in patients with PDR. A model was developed to predict the probability of PDR progression and measurement of the vitreous level of VEGF may be useful for predicting the outcome of surgery.

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Introduction

Proliferative diabetic retinopathy (PDR) is characterized by extensive neovascularization and vessel in growth into the vitreous body, with subsequent bleeding and scarring around the new vessels that leads to severe visual impairment.[1] Activity of PDR and outcome of vitreous surgery may be associated with several factors, but are inconclusive. Numerous cytokines and growth factors have been implicated in the pathogenesis and progression of PDR.[1] Vascular endothelial growth factor (VEGF) is an angiogenic and permeability-enhancing glycoprotein that predominantly acts on endothelial cells, and VEGF has been shown to increase in patients with active intraocular neovascularization.[1–3] Recently, it has been suggested that chronic subclinical inflammation may underlie much of the vascular pathology of diabetic retinopathy.[4] Interleukin-6 (IL-6) is a multifunctional cytokine[5] that may be a major mediator of anterior uveitis[6] and proliferative vitreoretinopathy (PVR).[7] IL-6 is related to hyperglycemia and hypoxia,[8] and it is considered to be an indirect inducer of angiogenesis that acts through the induction of VEGF.[9]

In an attempt to predict the outcome of vitreous surgery in PDR patients, we investigated whether vitreous and plasma levels of VEGF and/or IL-6 were correlated with the results, that is, whether it was possible to predict the outcome of surgery by measuring VEGF and IL-6 in the vitreous fluid obtained at operation along with assessment of fundus findings.

Materials and methods

Undiluted vitreous fluid samples were harvested at the start of vitrectomy after

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informed consent was obtained from each subject following an explanation of the purpose and potential adverse effects of the procedure. This study was performed in accordance with the 1975 Declaration of Helsinki, as revised in 1983 and the institutional review board (Tokyo Women's Medical University) also approved the protocol for collection of vitreous fluid and blood samples. Vitreous fluid samples were obtained from 73 patients with PDR. Vitreous bleeding may affect the measurement of VEGF, so we tried to avoid taking the vitreous haemorrhage during surgeries.[3] Those procedures were very difficult to implement completely in clinical cases. Vitrectomy was performed for vitreous and/or preretinal haemorrhage in 40 patients and for tractional retinal detachment with fibrous proliferation in 33 patients.

Pars plana vitrectomy was performed by a standardized technique involving three pars plana sclerotomy incisions. The vitreous body was removed as far as the vitreous base, followed by segmentation and delamination of proliferative membranes, removal of the posterior vitreous surface, and panretinal endolaser coagulation of the retina up to the ora serrata. If iatrogenic detachment from posterior breaks occurred, retinal detachment was treated with gas tamponade (25% SF6) at the end of vitreous surgery. All operations were performed at Tokyo Women's Medical University hospital. Exclusion criteria were: (1) prior ocular surgery, (2) a history of ocular inflammation, (3) retinal detachment associated with a retinal tear, and (4) rubeosis iridis or rubeotic glaucoma.

Preoperative, intraoperative, and postoperative fundus findings were recorded in each subject. The severity of diabetic retinopathy was assessed by standardized fundus colour photography and fluorescein angiography (FA), which were performed with a Topcon TRC-50IA fundus camera, an image-net system (Tokyo Optical Co Ltd., Japan), and a preset lens with a slit-lamp at 1 day before vitreous surgery and 6 months afterwards.[10] Diabetic retinopathy was graded according to the modified Early Treatment Diabetic Retinopathy Study (ETDRS) retinopathy severity scale.[10–12] In particular, the severity of new vessels elsewhere (NVE), new vessels on or within 1 disc diameter of the disc (NVD), fibrous proliferation elsewhere (FPE), vitreous haemorrhage, and retinal detachment were graded according to the ETDRS system.[11,12] Retinal photocoagulation was divided into three categories: grade 1 was 800 laser shots or less to the whole retina, grade 2 was 800–1600 shots or less, and grade 3 was 1600 shots or more. The severity of fundus findings in each photograph was graded to calculate the average severity.

Samples of undiluted vitreous fluid (0.3–0.7 ml) were aspirated under standardized conditions from directly above the retina at the beginning of surgery and were immediately transferred to sterile tubes and were rapidly frozen at –80°C. Plasma samples were also collected from the 73 patients. Blood was immediately placed on ice and subjected to centrifugation at 3000 g for 10 min at 4°C, after which the plasma was rapidly frozen at −80°C until assay.

Both VEGF and IL-6 were measured in vitreous samples from all eyes as well as in the plasma of each patient. Measurement was performed with an enzyme-linked immunosorbent assay (ELISA) for human VEGF or IL-6 (R&D System, Minneapolis, MN, USA).[10] The VEGF kit was able to detect two of the four isoforms of this factor ($VEGF_{121}$ and $VEGF_{165}$), probably because these two shorter isoforms are secreted and the longer isoforms are cell-associated. ELISA was performed according to the manufacturer's instructions. To determine the recovery rate of each ELISA, authentic VEGF or IL-6 was added to samples of vitreous fluid and plasma before measurement, and the recovery rate was found to be 89.8% for the VEGF assay and 95.2% for the IL-6 assay. When the precision of these ELISAs was assessed by repeated measurement of the same samples, the intraassay coefficient of variation (CV) was shown to be <7.5% for both assays. When VEGF and IL-6 levels in the vitreous fluid and plasma were assayed on five different days, the interassay CV was <8.9% for each sample. The levels of both factors in the vitreous fluid and plasma were generally within the detection range of these assays, since the minimum detectable concentration was 15.6 pg/ml for VEGF and 0.156 pg/ml for IL-6. Any concentrations below these levels were recorded as the minimum detectable concentration for statistical analysis.

The following systemic variables were measured at baseline. HbA_{1c} was measured by affinity chromatography (HPLC, Kyoto Chemical, Kyoto, Japan) (normal range: 4.3–5.8%). Systolic and diastolic blood pressures were measured with a mercury sphygomanometer in the sitting position after resting for 10 min. Hypertension was defined as a systolic blood pressure 140 mmHg or higher, a diastolic blood pressure of 90 mmHg or higher, or treatment with antihypertensive medication. The urinary albumin concentration was measured by an immunoturbidmetric method, with a reference of less than 12.0 mg/g creatinine. Microalbuminuria was defined as a urinary albumin concentration of 12 mg/g creatinine or more, and proteinuria was defined as a concentration 300 mg/dl or more.

Analyses were performed with SAS System 8e software (SAS Institute Inc., Cary, NC, USA).[13] Results are presented as the mean±SD or as the geometric mean±SD for logarithmic data. To assess the

relationship between each angiogenic factor and the ETDRS retinopathy severity, Spearman's rank-order correlation coefficients were calculated. Odds ratios were calculated using a logistic regression model with three dummy variables. To identify independent predictors of the progression of PDR, univariate and multivariate logistic regression analyses were performed by the vest-subset variables selection method. Two-tailed probability values of <0.05 were considered to indicate statistical significance.

Results

Complete data were available for 73 out of 81 patients, while eight patients were lost during follow-up because of transfer of care to another hospital or nonattendance at the outpatient clinic. The clinical characteristics of subjects with PDR were showed in Table 1. All patients were followed up for 6 months. The baseline PDR grade was showed in Table 2. At the end of follow-up, PDR showed no improvement and/or progression by 1 level or more in 23 eyes (32%), while it regressed by 1 level or more in 50 eyes (68%) (Table 2). In all, 52 patients noted a significant improvement of visual acuity after surgery, 62 patients (85%) achieved a visual acuity of 20/200 or better, and 46 patients (63%) had vision of 20/40 or better. The remaining 11 patients (15%) showed a decline of visual acuity; four had diabetic macular oedema, four had vitreous haemorrhage, and three had ischaemic maculopathy and/or macular atrophy. Intraoperative retinal photocoagulation was carried out to a total of 768.4 shots in patients with grade 1 photocoagulation preoperatively, while patients who were grade 2 preoperatively received 472.3 shots and grade 3 patients received 211.6 shots.

Table 1 Clinical characteristics of the subjects

Characteristic	No. (%) or Mean $\pm$ SD (range) (Total n = 73)
Age (years)	63.6 ± 10.5 (37–76)
Gender	
Male	46 (63)
Female	27 (37)
Duration of DM	15.2 ± 7.5 (1–28)
HbA_{1c} (%)	7.5 ± 1.6 (5.1–11.4)
Laser photocoagulation	
Grade 1	28 (38)
Grade 2	30 (41)
Grade 3	15 (21)

DM: diabetes mellitus; HbA_{1c}: hemoglobin A_{1c}.

Table 2 Severity of diabetic retinopathy at baseline and 6 months after surgery

Baseline Severity	Severity after 6 months						
	47	53	61	71	81	85	Total
71	3	6	5	9	4	0	27
75	3	5	1	2	2	0	13
81	0	6	4	5	4	1	20
85	0	4	3	3	0	3	13
Total	6	21	13	19	10	4	73

ETDRS retinopathy severity score are shown.

Vitreous fluid levels of VEGF were significantly higher in the patients with no improvement and/or progression of PDR (1789.2 pg/ml (198.5–3436.8)) than in the patients with regression (711.8 pg/ml (68.4–1968.1)) ($P = 0.0032$). Vitreous fluid levels of IL-6 were also significantly higher in the patients with no improvement and/or progression of PDR (347.2 pg/ml (26.2–758.6)) than in the patients with regression of PDR (85.3 pg/ml (12.6–398.2)) ($P = 0.0088$). On the other hand, there was no significant difference of plasma VEGF levels between no improvement and/or progression of PDR (73.7 pg/ml (15.6–242.7)) and regression of PDR (78.2 pg/ml (15.6–276.4)) ($P = 0.5124$). There was also no significant difference of plasma IL-6 levels between no improvement and/or progression of PDR (25.6 pg/ml (4.0–130.4)) and regression of PDR (24.2 pg/ml (4.0–142.3)) ($P = 0.4907$).

Vitreous levels of VEGF were also significantly correlated with the severity of NVE, NVD, and FPE, but not with the severity of vitreous haemorrhage and retinal detachment (Table 3). Vitreous levels of IL-6 were significantly correlated with the severity of NVE, NVD, and FPE, but were not significantly correlated with the severity of vitreous haemorrhage and retinal detachment. On the other hand, plasma level of VEGF was not significantly correlated with severity of NVE, NVD, vitreous haemorrhage, FPE, and retinal detachment. Plasma level of IL-6 was also not significantly correlated with those.

Vitreous levels of both VEGF and IL-6 were significantly correlated with the baseline extent of retinal photocoagulation (VEGF; $\rho = -0.6882$, $P < 0.0001$: IL-6; $\rho = -0.4762$, $P = 0.0078$). The vitreous level of VEGF was significantly correlated with the severity of diabetic retinopathy ($\rho = 0.648$, $P < 0.0001$, Figure 1a) and the vitreous level of IL-6 was also significantly correlated with the severity of diabetic retinopathy ($\rho = 0.596$, $P < 0.0001$, Figure 1b).

Univariate logistic regression analysis using the number of patients and the above-mentioned factors showed that the vitreous levels of IL-6 and VEGF, as well

Table 3 Correlation between fundus finding grade and vitreous and plasma levels of VEGF and IL-6

| Characteristics | Vitreous fluid level | | | | Plasma level | | | |
| | VEGF | | IL-6 | | VEGF | | IL-6 | |
	ρ	P-value	ρ	P-value	ρ	P-value	ρ	P-value
NVE	0.6002	0.0048	0.5644	0.0090	0.1274	0.3342	0.0934	0.4876
NVD	0.7104	0.0008	0.5932	0.0060	0.1471	0.3106	0.1146	0.4283
FPE	0.5424	0.0098	0.3218	0.0226	0.2416	0.0784	0.1984	0.1136
Vitreous haemorrhage	0.2114	0.1243	0.1021	0.4262	0.2036	0.0910	0.2002	0.1033
Retinal detachment	0.1982	0.1616	0.1425	0.2298	0.2264	0.0832	0.2138	0.8943

VEGF: vascular endothelial growth factor; IL-6: intereukin-6; NVE: new vessels elsewhere; NVD: new vessels on or within 1 disc diameter of the disc; FPE: fibrous proliferation elsewhere.

as the severity of NVE, NVD, FPE, the amount of preoperative laser shots, and the amount of operative laser shots, were associated with the progression of PDR (Table 4). In contrast, there was no significant relationship between the progression of PDR and the plasma levels of IL-6 or VEGF, and the severity of vitreous haemorrhage or TRD.

Multivariate logistic regression analysis confirmed that VEGF was a significant predictor of the progression of PDR (Odds ratio (95% confidence interval): 2.72 (1.06–6.38), $P = 0.0003$) (Table 5). Based on the estimated coefficients of PDR, a formula was devised to allow calculation of changes in the risk of PDR progression based on the quantitative values of risk factors such as VEGF: estimated probability of progression of $PDR = 1/\{1 + \exp(+6.398 - 1.716 \times \log 10(VEGF))\}$.

The vitreous fluid concentration of VEGF was significantly higher than the plasma VEGF level (75.9 pg/ml (15.6–276.4)) in the patients with PDR ($P < 0.0001$). The vitreous fluid concentration of IL-6 was also significantly higher than the plasma IL-6 level (24.6 pg/ml (4.0–142.3)) in the PDR patients ($P = 0.0076$). There was no significant relationship between the vitreous fluid level of VEGF and age, duration of diabetes, HbA_{1c}, hypertension, or nephropathy ($P = 0.3719$, $P = 0.3145$, $P = 0.2143$, $P = 0.2657$, and $P = 0.4391$, respectively). There was also no significant relationship between the vitreous fluid levels of IL-6 and these factors ($P = 0.4811$, $P = 0.2836$, $P = 0.3042$, $P = 0.2891$, and $P = 0.2962$, respectively).

Discussion

In this prospective study, we investigated whether measurement of the vitreous and plasma levels of VEGF (an angiogenic factor) and/or IL-6 (a proinflammatory cytokine) was useful to predict the progression of PDR along with assessment of fundus findings and the outcome of vitreous surgery in PDR patients. The

vitreous fluid levels of both VEGF and IL-6 were significantly elevated in patients who showed no improvement and/or progression of PDR when compared with the levels in patients who showed regression of PDR. Vitreous levels of VEGF and IL-6 were significantly correlated with the severity of fundus findings such as NVE, NVD, and FPE, all of which are related to neovascularization and activity of PDR. There was no significant difference of plasma levels of both VEGF and IL-6 between no improvement and/or progression of PDR and regression of PDR. The plasma levels of both VEGF and IL-6 were not correlated with severity of fundus findings. Furthermore, the vitreous fluid levels of VEGF and IL-6 were significantly higher than the plasma levels of those. Accordingly, our results suggested that the progression of PDR and the outcome of vitreous surgery were correlated with the vitreous levels of VEGF and/or IL-6, implying that an increase of intraocular VEGF and/or IL-6 production induced more active neovascularization.

It has been reported that VEGF is expressed by a number of cells in the eye, such as retinal pigment epithelial cells, pericytes, endothelial cells, Müller cells, and astocytes,[14,15] and that intraocular synthesis is the main factor contributing to the high-vitreous concentration of VEGF in PDR.[2,3] It has been suggested that IL-6 may indirectly induce angiogenesis by stimulating VEGF expression, as occurs with the induction of VEGF mRNA by hypoxia.[9] It has been suggested that induction of VEGF mRNA probably occurs through transcriptional regulation, so previous reports combined with our results would indicate that IL-6 may affect neovascularization and the progression of PDR after vitreous surgery by upregulating expression of VEGF. However, further investigations will be needed to clarify the ocular interactions between IL-6 and VEGF, as well as the role of IL-6 during neovascularization in PDR patients and after vitreous surgery.
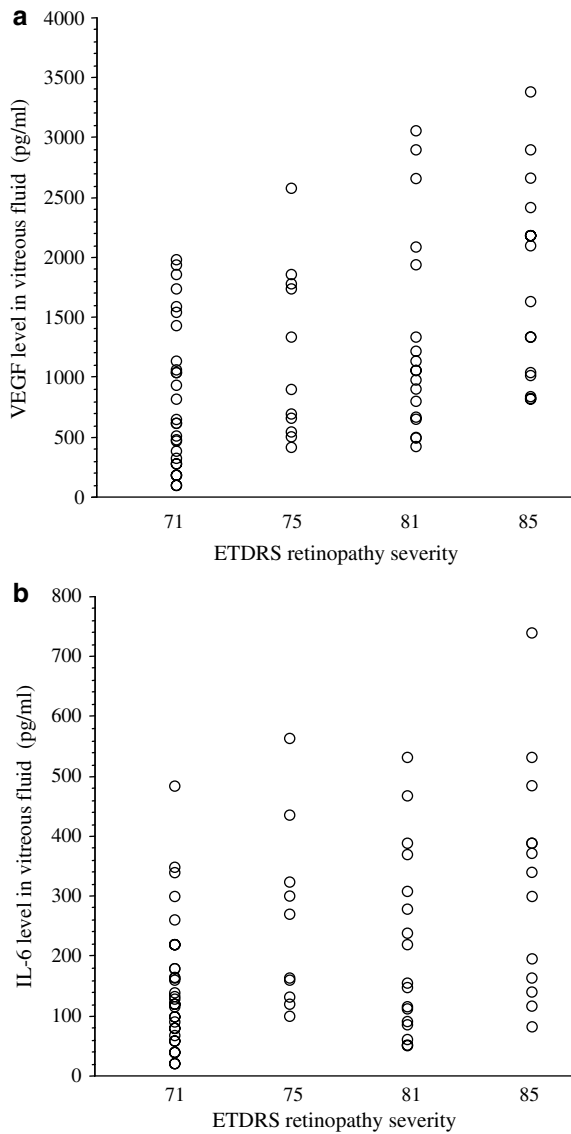
Figure 1 (a) Correlation between the severity of diabetic retinopathy and the vitreous level of VEGF. The severity of retinopathy was evaluated using the ETDRS retinopathy severity scale. VEGF levels in vitreous fluid were significantly correlated with the severity of diabetic retinopathy ($\rho = 0.648$, $P < 0.0001$). (b) Correlation between the severity of diabetic retinopathy and the vitreous level of IL-6. Vitreous fluid levels of IL-6 were significantly correlated with the severity of diabetic retinopathy ($\rho = 0.596$, $P < 0.0001$).

Table 4 Univariate logistic regression analysis of factors related to the progression of proliferative diabetic retinopathy

Variable	Odds ratio (95% CI)	P-value
NVE	1.86 (1.03–3.06)	0.0166
NVD	2.16 (1.06–3.78)	0.0098
FPE	1.71 (1.01–2.52)	0.0219
Vitreous haemorrhage	1.20 (0.58–1.94)	0.1423
TRD	1.30 (0.62–2.32)	0.0827
Vitreous VEGF (log10 pg/ml)	3.12 (1.08–6.27)	0.0006
Vitreous IL-6 (log10 pg/ml)	2.27 (1.04–4.60)	0.0084
Plasma VEGF (log10 pg/ml)	1.18 (0.54–1.72)	0.1783
Plasma IL-6 (log10 pg/ml)	1.06 (0.48–1.61)	0.1974
Preoperative laser shots	0.56 (0.22–0.88)	0.0382
Operative laser shots	1.32 (0.78–1.84)	0.0482

CI, confidence interval; NVE, new vessels elsewhere; NVD, new vessels on or within 1 disc diameter of the disc; FPE, fibrous proliferation elsewhere; TRD, tractional retinal detachment; VEGF, vascular endothelial growth factor; IL-6, interleukin-6.

Table 5 Multivariate logistic regression analysis of factors related to the progression of proliferative diabetic retinopathy

Variable	Odds ratio (95% CI)	P-value
VEGF (log10 pg/ml)	2.72 (1.06–6.38)	0.0003

CI, confidence interval; VEGF, vascular endothelial growth factor.

We used logistic regression analysis to identify independent risk factors associated with the progression of PDR after vitreous surgery. It is possible that the lack of a correlation between certain factors and the success or failure of surgery may be related to an inadequate sample size. However, our univariate logistic regression analysis showed that several factors were associated with the progression of PDR. These factors included the vitreous levels of VEGF and IL-6, as well as the severity of NVE, NVD, FPE, amount of preoperative laser shots, and amount of operative laser shots. All three fundus parameters (NVE, NVD, and FPE) reflect retinal neovascularization, while VEGF and IL-6 are involved in the onset and progression of new vessel formation.[1,2,9] It was suggested that the additional laser was needed to reduce the activity of new vessel formation during surgery in the advanced cases. Therefore, active neovascularization is associated with the progression of PDR. The advantage of this prospective study was to investigate the correlation between predicting factors and/or risk factors and the outcome of vitreous surgery by performing multivariate analyses. Multivariate logistic regression analysis also showed that the vitreous level of VEGF alone was associated with the progression of PDR, but the vitreous level of IL-6 and the various fundus parameters (including the severity of NVE, NVD, FPE, and amount of preoperative laser shots) did not show a significant association with the progression of PDR. The reason why the vitreous level of IL-6 and the severity of NVE, NVD, or FPE, and amount of preoperative laser shots were not selected as risk factors

for the progression of PDR by multivariate analysis is that all these factors were significantly associated with the vitreous level of VEGF, which thus seems to be the most important risk factor among the picked-up candidates.[1,2] For each 100 pg/ml increase in VEGF level in the vitreous, the odds of progression of postoperative PDR were increased by 2.72 times. Based on the results of this study, a simple model was developed to predict the probability of PDR progression in clinical severity. The results and our formula are the trial at present, and the advanced studies, including many cases in multicenter study, is mandatory to develop the useful formula to predict the progression of PDR and the outcome of vitreous surgery in PDR patients. Then, as a result of surgery, the vitreous composition is altered, for examples, levels of other cytokines or as yet unidentified factors change. This, in combination with high levels of VEGF, triggers the progression of postoperative PDR.

In conclusion, the vitreous levels of VEGF and/or IL-6 were correlated with the progression of PDR and the outcome of vitreous surgery. This information can be the first step to develop the predictor for vitreous surgery outcome. A model was developed to predict the probability of PDR progression and measurement of the vitreous level of VEGF may be useful for predicting the outcome of surgery.

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