EXTENDED REPORT

Risk evaluation of outcome of vitreous surgery for proliferative diabetic retinopathy based on vitreous level of vascular endothelial growth factor and angiotensin II

H Funatsu, H Yamashita, H Noma, T Mimura, K Sakata, S Hori

Aims: To ascertain whether measurement of the vitreous fluid levels of vascular endothelial growth factor (VEGF) or angiotensin II (Ang II) could predict the outcome of vitreous surgery in patients with proliferative diabetic retinopathy (PDR).

Methods: A prospective observational case study was performed in 61 consecutive patients (61 eyes) with PDR who underwent vitreoretinal surgery. Vitreous fluid samples were obtained during surgery. The VEGF level in vitreous fluid and plasma was determined by enzyme linked immunosorbent assay, while the Ang II level was measured by radioimmunoassay. 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 was seen in 15 (25%) of the 61 eyes. Vitreous levels of VEGF and Ang II were significantly higher in eyes with progression of PDR than in eyes with regression of PDR (p = 0.0044, and p = 0.0178, respectively). Multivariate logistic regression analysis showed that the vitreous VEGF level increased along with the progression of PDR after vitreous surgery (odds ratio 2.48, p = 0.008).

Conclusion: A high vitreous fluid VEGF level is associated with a significant risk of postoperative progression of PDR. The vitreous level of VEGF at the time of surgery may be a useful predictor of the outcome.

The outcome of vitreous surgery for proliferative diabetic retinopathy (PDR) has improved during the past decade because the instruments and the surgical technique have been refined. The initial indications and rationale for vitreous surgery were largely established on the basis of fundus findings such as new vessels elsewhere (NVE), new vessels on or within 1 disc diameter of the disc (NVD), fibrous proliferation elsewhere (FPE), vitreous haemorrhage, and tractional retinal detachment. However, it now seems to be necessary to predict the outcome of vitreous surgery, because extensive neovascularisation can occur after surgery for PDR.

Recently, various cytokines have been suggested to play a part in the pathogenesis and progression of diabetic retinopathy. To assess the clinical significance of monitoring cytokines or growth factors, such as vascular endothelial growth factor (VEGF), interleukin 6 (IL-6), and endostatin, we have been investigating the correlations between these peptides and the progression of PDR and/or diabetic macular oedema (DMO). We previously reported that both the level of angiotensin II (Ang II), a vasoactive factor, and that of VEGF, a stimulator of angiogenesis, were significantly higher in the vitreous fluid of patients with PDR than in that of non-diabetic patients or diabetic patients without retinopathy, and that the levels of both Ang II and VEGF were elevated in the active stage of PDR compared with in the quiescent stage. These findings suggest that Ang II may contribute to the development and progression of PDR together with VEGF.

In an attempt to predict the prognosis of PDR patients undergoing vitreous surgery, we investigated whether the vitreous levels of Ang II and VEGF were correlated with the outcome—that is, whether it was possible to predict the outcome of surgery by measuring Ang II and VEGF in vitreous fluid obtained at operation, along with assessment of fundus findings.

PATIENTS AND METHODS

Patients

Undiluted vitreous fluid samples were harvested at the start of vitrectomy after informed consent was obtained from each subject following explanation of the purpose and potential adverse effects of the procedure. This study was performed in accordance with the Helsinki Declaration of 1975 (the 1983 revision), and the institutional review board of Tokyo Women's Medical University also approved the protocol for collection of vitreous fluid and blood samples. Vitreous fluid samples were obtained from 61 patients with PDR. Vitrectomy was performed to treat vitreous haemorrhage and/or preretinal haemorrhage in 33 patients, while it was done for tractional retinal detachment associated with fibrovascular proliferation in 28 patients.

Pars plana vitrectomy was done by a standard technique using three pars plana sclerotomy incisions. Undiluted samples of vitreous fluid (0.3–0.7 ml) were aspirated under standardised conditions from directly in the mid-vitreous at the beginning of surgery and were immediately transferred to sterile tubes. After collection of the vitreous fluid samples, the vitreous was removed as far as its base, followed by segmentation and delamination of proliferative membranes, removal of the posterior vitreous surface, and panretinal

Abbreviations: ACE, angiotensin converting enzyme; Ang II, angiotensin II; CV, coefficient of variation; ELISA, enzyme linked immunosorbent assay; FPE, fibrous proliferation elsewhere; NVD, new vessels on or within 1 disc diameter of the disc; NVE, new vessels elsewhere; PDR, proliferative diabetic retinopathy; RIA, radioimmunoassay; TRD, tractional retinal detachment; VEGF, vascular endothelial growth factor.
The blood was immediately placed on ice and centrifuged at 2000 rpm for 10 minutes. The separated serum was stored at −80°C until assay.

Preoperative, intraoperative, and postoperative fundus findings were recorded for each subject. The severity of diabetic retinopathy was assessed by standardised fundus photography and fluorescein angiography, and a preset lens was used to obtain a detailed image of the retina. Three fundus photographs were taken at 1 day before and 6 months after vitreous surgery. The extent of retinal photocoagulation was classified based on the modified Early Treatment Diabetic Retinopathy Study (ETDRS) system.”

The changes of fundus findings were assessed using the ETDRS severity score. The severity of fundus findings was graded in each photograph and the average severity was calculated. The severity of diabetic retinopathy was compared between before and 6 months after vitreous surgery.

Statistical analysis
Analyses were performed with SAS System 8e software (SAS Institute Inc, Cary, NC, USA). Results are presented as the mean (SD) or as the geometric mean (SD) for logarithmic data. To identify independent predictors of the progression of PDR, univariate and multivariate logistic regression analyses were performed by the best subset variables selection method. To assess the relation between each factor tested and the severity of retinopathy, Spearman’s rank order correlation coefficients were calculated. Odds ratios were calculated using a logistic regression model with three dummy variables. A probability value (two tailed) of less than 0.05 was considered to indicate statistical significance.

RESULTS
Baseline patient profile
Complete data were available for 61 out of 67 patients, while six patients were lost to follow up because of transfer to another hospital or non-attendance at our outpatient clinic. Of the 61 remaining patients, 35 were men and 26 were women (table 1). Their mean age was 62.4 years (range 39–78 years), the mean duration of diabetes was 14.8 years (range 2–30 years), and the mean HbA1c level was 7.6% (range 5.0–10.6%). These 61 patients were all followed for at least 6 months. The baseline PDR grade was level 71 in 20 eyes, level 75 in 13 eyes, level 81 in 17 eyes, and level 85 in 11 eyes (table 2). Panretinal photocoagulation had been performed before vitreous surgery in 37 patients and focal retinal photocoagulation had been done in 19 patients, while five patients had not undergone preoperative retinal photo-coagulation.

Outcome of vitreous surgery
At the end of follow up, PDR showed no improvement and/or progression by one level or more in 15 patients (25%), while there was regression by one level or more in 46 patients (75%) (table 2). Forty two patients noted a significant improvement of visual acuity after surgery and 49 patients (80%) achieved a visual acuity of 20/200 or better. Among the remaining 12 patients (20%) with worsening of their visual acuity, four had diabetic macular oedema, four had vitreous haemorrhage, and four had macular atrophy.

Table 1: Clinical and laboratory characteristics of the patients

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>No.</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (years)</td>
<td>61</td>
<td>62.4 (9.6)†</td>
</tr>
<tr>
<td>Sex</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>35</td>
<td>57.4%</td>
</tr>
<tr>
<td>Female</td>
<td>26</td>
<td>42.6%</td>
</tr>
<tr>
<td>Duration of diabetes (years)</td>
<td>61</td>
<td>14.8 (7.3)†</td>
</tr>
<tr>
<td>Treatment:</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Diet</td>
<td>6</td>
<td>9.8%</td>
</tr>
<tr>
<td>Oral hypoglycaemic agents</td>
<td>33</td>
<td>54.1%</td>
</tr>
<tr>
<td>Insulin</td>
<td>22</td>
<td>36.1%</td>
</tr>
<tr>
<td>HbA1c [%]</td>
<td>61</td>
<td>7.6 (2.2)†</td>
</tr>
<tr>
<td>Plasma VEGF concentration (pg/ml)</td>
<td>61</td>
<td>6.2 (30.7)†</td>
</tr>
<tr>
<td>Plasma Ang II concentration (pg/ml)</td>
<td>61</td>
<td>12.9 (7.5)†</td>
</tr>
<tr>
<td>Vitreous VEGF concentration (pg/ml)</td>
<td>61</td>
<td>1.2615 (1094.4)†</td>
</tr>
<tr>
<td>Vitreous Ang II concentration (pg/ml)</td>
<td>61</td>
<td>21.8 (16.2)†</td>
</tr>
</tbody>
</table>

*Numbers show the number of patients with available data. †Mean (SD).

HbA1c = haemoglobin A1c; VEGF = vascular endothelial growth factor; Ang II = angiotensin II.
retinal photocoagulation (VEGF; \( r = 0.35996, p = 0.0146 \)) and regression of PDR (4.0–22.1) (\( p = 0.0178 \)). On the other hand, there was no significant difference of plasma VEGF levels between no improvement and/or progression of PDR (35.8 pg/ml (4.0–22.1)) and regression of PDR (65.8 pg/ml (15.6–218.5)) and regression of PDR (62.7 pg/ml (15.6–206.5)) (\( p = 0.5972 \).)

There was also no significant difference of plasma Ang II levels between no improvement and/or progression of PDR (13.8 pg/ml (4.0–23.4)) and regression of PDR (12.2 pg/ml (4.0–25.2)) (\( p = 0.5329 \)).

The vitreous fluid level of VEGF was significantly correlated with severity of NVE, NVD, and FPE (NVE; \( r = 0.18146, p = 0.3027 \); NVD; \( r = 0.20165, p = 0.1832 \)) and severity of vitreous haemorrhage or retinal detachment (vitreous haemorrhage; \( r = 0.1309, p = 0.3146 \); retinal detachment; \( r = 0.20165, p = 0.1832 \)). The vitreous level of Ang II was also significantly correlated with severity of NVE, NVD, and FPE (NVE; \( r = 0.38926, p = 0.0138 \); NVD; \( r = 0.33667, p = 0.0264 \); FPE; \( r = 0.27643, p = 0.0486 \)), but not with severity of vitreous haemorrhage or retinal detachment (vitreous haemorrhage; \( r = 0.13246, p = 0.3782 \); retinal detachment; \( r = 0.19984, p = 0.1744 \)). On the other hand, plasma levels of VEGF were not significantly correlated with severity of NVE, NVD, vitreous haemorrhage, FPE, and retinal detachment (NVE; \( r = 0.1441, p = 0.3027 \); NVD; \( r = 0.1274, p = 0.3356 \); vitreous haemorrhage; \( r = 0.0974, p = 0.4726 \); FPE; \( r = 0.1309, p = 0.3146 \); retinal detachment; \( r = 0.0883, p = 0.5124 \)). Plasma levels of Ang II were also not significantly correlated with those (NVE; \( r = 0.1193, p = 0.3904 \); NVD; \( r = 0.1038, p = 0.4398 \); vitreous haemorrhage; \( r = 0.0902, p = 0.5516 \); FPE; \( r = 0.1298, p = 0.3217 \); retinal detachment; \( r = 0.0744, p = 0.5761 \)).

Outcome of vitreous surgery and risk factors

Univariate logistic regression analysis using the number of patients and the above mentioned factors showed that the vitreous levels of VEGF and Ang II, as well as the severity of NVE, NVD, and FPE, were associated with the progression of PDR (table 3).

Multivariate logistic regression analysis confirmed that VEGF was a significant predictor of the progression of PDR (table 4). 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 changes of risk factors such as VEGF: estimated probability of progression of PDR = \( 1/(1+\exp[+5.842 - 1.562 \times \log10(VEGF)]) \).

Stratification of retinopathy progression by VEGF and Ang II level

The patients are stratified into four groups based on the median vitreous fluid levels of VEGF and Ang II (median VEGF level: 1120 pg/ml, median Ang II level: 25.0 pg/ml). In group 1 (low levels of both VEGF (<1120 pg/ml) and Ang II (<25.0 pg/ml)), 30 patients (94%) showed improvement of PDR and two patients (6%) showed progression. In group 2 (a high VEGF level (≥1120 pg/ml) and a low Ang II level (<25.0 pg/ml)), five out of eight patients (62%) showed improvement and three patients (38%) showed progression. In group 3 (a high Ang II level (≥25.0 pg/ml) and a low

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

<table>
<thead>
<tr>
<th>Severity at baseline*</th>
<th>Severity after 6 months*</th>
</tr>
</thead>
<tbody>
<tr>
<td>47</td>
<td>53</td>
</tr>
<tr>
<td>61</td>
<td>71</td>
</tr>
<tr>
<td>81</td>
<td>85</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td><strong>Total</strong></td>
</tr>
</tbody>
</table>

*ETDRS retinopathy severity score.

Table 3 Univariate logistic regression analysis of factors related to the progression of proliferative diabetic retinopathy

<table>
<thead>
<tr>
<th>Variable</th>
<th>Odds ratio (95% CI)</th>
<th>p Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>NVE</td>
<td>1.98 (1.11 to 3.44)</td>
<td>0.0098</td>
</tr>
<tr>
<td>NVD</td>
<td>1.42 (1.06 to 2.02)</td>
<td>0.0072</td>
</tr>
<tr>
<td>FPE</td>
<td>1.71 (1.01 to 2.52)</td>
<td>0.0219</td>
</tr>
<tr>
<td>Vitreous haemorrhage</td>
<td>1.32 (0.74 to 1.98)</td>
<td>0.4128</td>
</tr>
<tr>
<td>TRD</td>
<td>1.58 (1.02 to 2.32)</td>
<td>0.0892</td>
</tr>
<tr>
<td>Vitreous VEGF (log 10 pg/ml)</td>
<td>2.24 (1.08 to 3.58)</td>
<td>0.0012</td>
</tr>
<tr>
<td>Vitreous Ang II (log 10 pg/ml)</td>
<td>1.76 (1.18 to 2.43)</td>
<td>0.0136</td>
</tr>
<tr>
<td>Plasma VEGF (log 10 pg/ml)</td>
<td>1.14 (0.48 to 1.68)</td>
<td>0.1968</td>
</tr>
<tr>
<td>Plasma Ang II (log 10 pg/ml)</td>
<td>1.06 (0.42 to 1.73)</td>
<td>0.2014</td>
</tr>
</tbody>
</table>

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; Ang II = angiotensin II.
VEGF level (<1120 pg/ml)), two out of six patients (25%) showed progression of PDR, while eight out of 15 patients (53%) showed progression in group 4 (high levels of both VEGF (≥1120 pg/ml) and Ang II (≥25.0 pg/ml)). Groups 2, 3, and 4 had a significantly higher risk of PDR progression after vitreous surgery (group 2 odds ratio: 3.98, \( p = 0.0316 \), group 3 odds ratio: 2.46, \( p = 0.0482 \), group 4 odds ratio: 7.19, \( p = 0.0019 \)) (table 5).

### DISCUSSION

In this prospective study, we investigated whether the outcome of vitreous surgery for PDR was correlated with the vitreous fluid levels of VEGF and/or Ang II. We found that vitreous fluid levels of both VEGF and Ang II were significantly higher in patients who showed no improvement and/or progression of PDR when compared with the levels in patients who showed progression of PDR. The vitreous levels of VEGF and Ang II were also significantly correlated with the severity of NVE, NVD, and FPE, but not with the severity of vitreous haemorrhage or retinal detachment.

Various studies have shown that the vitreous levels of certain cytokines and growth factors are correlated with the severity of PDR.\(^{14-17}\) However, there have only been a few prospective studies on the prognosis of PDR and/or proliferative vitreoretinopathy (PVR).\(^{20-21}\) and previous attempts at predicting the prognosis have not been very successful. Although vitreous surgery for PDR has been established for over 10 years, many patients still do not achieve a good outcome.\(^1\) The main causes of a poor outcome after vitreous surgery are rheumatic glaucoma and tractional retinal detachment with fibrous vascular proliferation, so the prognosis seems to depend on the extent of neovascularisation. It is important to develop a method for predicting the outcome of vitreous surgery of PDR since this will help to improve the prognosis of surgical treatment. Measurement of VEGF and Ang II levels in the vitreous fluid may be useful in this respect, because both of these factors are correlated with neovascularisation. From our results, the plasma levels of VEGF or Ang II cannot be used to predict the outcome of vitreous surgery, because there was no significant difference in plasma levels of VEGF or Ang II between no improvement and/or progression of PDR; regression of PDR and the plasma levels of VEGF and Ang II were not significantly correlated with severity of fundus parameters.

In this prospective study, univariate logistic regression analysis showed that several factors were associated with the progression of PDR. These factors included the vitreous levels of VEGF and Ang II, as well as the severity of NVE, NVD, and FPE. All three fundus parameters reflect retinal neovascularisation, while VEGF and Ang II are involved in the onset and progression of new vessel formation.\(^4\) Therefore, a high level of neovascularisation is associated with the progression of PDR after vitreous surgery. Multivariate logistic regression analysis also showed that the vitreous level of VEGF was associated with the progression of PDR, while the vitreous level of Ang II and the fundus parameters no longer showed a significant association with PDR. The reason why the vitreous level of Ang II and the severity of NVE, NVD, or FPE were not selected as risk factors is that all these factors are significantly associated with the vitreous level of VEGF, which seems to be the most powerful risk factor. These results suggest that measurement of VEGF in the vitreous fluid may be useful for predicting the outcome of vitreous surgery for PDR. However, our sample size was relatively small, so a larger prospective study is required to confirm the predictive value of VEGF (or Ang II).

We have prospectively investigated the expression of VEGF and Ang II in the vitreous fluid of patients with PDR who underwent a primary vitrectomy. No improvement (containing the progression group) and regression of PDR was stratified into four groups according to the vitreous levels of VEGF and Ang II. The eyes with not only high levels of VEGF or Ang II but also both high levels of VEGF and Ang II in the vitreous fluid had a significantly high risk of progression of PDR in comparison with the eyes with low levels of both VEGF and Ang II. The advantage of this prospective study was the ability to perform a logistic regression model and estimated the odds ratio of postoperative progression according to the vitreous levels of VEGF and Ang II. Our results suggested that there is an interaction between VEGF and Ang II in the eye and the increase of both VEGF and Ang II production induces the activity of neovascularisation in patients with PDR.

Predicting the outcome of vitreous surgery for PDR is clinically important, but is not always easy. Stereoscopic fundus examination, fundus colour photography, FA, and examination using a preset lens with a slit lamp are all methods employed to diagnose the severity and activity of PDR. However, these clinical examinations are of limited value for predicting the outcome of vitreous surgery, probably because neovascularisation activity is not only dependent on fundus findings but also on the intraocular levels of mediators that promote new vessel formation and proliferation. We found that vitreous levels of VEGF and Ang II were higher in patients with active PDR than in patients with quiescent PDR.\(^3\) This suggests that measurement of the

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

<table>
<thead>
<tr>
<th>Variable</th>
<th>Odds ratio (95% CI)</th>
<th>p Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>VEGF (log 10 pg/ml)</td>
<td>2.48 (1.02 to 4.98)</td>
<td>0.0008</td>
</tr>
</tbody>
</table>

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

#### Table 5 Odds ratios for groups based on the vitreous fluid levels of VEGF and Ang II

<table>
<thead>
<tr>
<th>ODds ratio (95% CI)</th>
<th>p Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Group 1</td>
<td>1.00</td>
</tr>
<tr>
<td>Group 2</td>
<td>3.98 (0.78 to 16.5)</td>
</tr>
<tr>
<td>Group 3</td>
<td>2.46 (0.66 to 12.8)</td>
</tr>
<tr>
<td>Group 4</td>
<td>7.19 (1.03 to 47.04)</td>
</tr>
</tbody>
</table>

CI = confidence interval; group 1, VEGF<1120 pg/ml and Ang II<25.0 pg/ml; group 2, VEGF≥1120 pg/ml and Ang II<25.0 pg/ml; group 3, VEGF<1120 pg/ml and Ang II≥25.0 pg/ml; group 4, VEGF≥1120 pg/ml and Ang II≥25.0 pg/ml.
vitreous level of VEGF and/or Ang II may be useful to predict neovascularisation activity even when patients have already undergone retinal photocoagulation, and this test may possibly reduce the risk of visual disturbance caused by surgery for PDR.

In conclusion, this study revealed that the vitreous level of VEGF was correlated with the progression of PDR and was elevated when PDR showed progression. These findings suggest that VEGF (an angiogenesis stimulator) may determine whether progression or regression of PDR occurs after vitreous surgery. We also used these results to develop an equation for predicting the risk of postoperative progression of PDR. A high vitreous fluid VEGF level was a significant risk factor for and a potential predictor of the postoperative progression of PDR. Advances in diagnostic techniques may allow the intraoperative measurement of VEGF in the vitreous fluid and help to improve prediction of the outcome of vitreous surgery for PDR. However, a large scale clinical study is needed to determine whether the measurement of VEGF is actually useful or not.

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Authors' affiliations
H Funatsu, Department of Ophthalmology, Diabetes Center, Tokyo Women’s Medical University, Japan
H Yamashita, Department of Ophthalmology and Visual Science, Yamagata University School of Medicine, Japan
H Noma, Department of Ophthalmology and Visual Science, Hiroshima University School of Medicine, Japan
T Mimura, Department of Ophthalmology, Tokyo University School of Medicine, Japan
K Sakata, S Hori, Department of Ophthalmology, Tokyo Women’s Medical University, Japan

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