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CLINICAL STUDY

Aqueous humour levels of cytokines are correlated to vitreous levels and severity of macular oedema in branch retinal vein occlusion

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Abstract

Aim **To investigate whether the aqueous levels of vascular endothelial growth factor (VEGF) and interleukin-6 (IL-6) are correlated to the vitreous levels of these substances and to the severity of macular oedema in branch retinal vein occlusion (BRVO).**

Methods **Aqueous and vitreous samples were obtained during cataract and vitreous surgery from 24 patients (24 eyes) with macular oedema in BRVO. The VEGF and IL-6 levels in aqueous humour, vitreous fluid, and plasma were determined by enzyme-linked immunosorbent assay. The degree of retinal ischaemia was evaluated in terms of the area of capillary nonperfusion using the Scion Image. The severity of macular oedema was evaluated using the OCT.**

Results **The aqueous level of VEGF was significantly correlated with the vitreous level of VEGF ($P<0.0001$). Vitreous levels of VEGF and IL-6 were significantly correlated with the nonperfusion area of BRVO ($P<0.0001$, $P=0.0061$, respectively), as were the aqueous levels of VEGF and IL-6 ($P<0.0001$, $P=0.0267$, respectively). Furthermore, the vitreous levels of VEGF and IL-6 and the aqueous level of VEGF were significantly correlated with the severity of macular oedema of BRVO ($P=0.0001$, $P=0.0331$, $P=0.0272$, respectively).**

Conclusion **Our results suggest that the aqueous level of VEGF may reflect its vitreous level. Measurement of the aqueous level of VEGF may be clinically useful to indicate the severity of macular oedema with BRVO.**

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Keywords: branch retinal vein occlusion; macular oedema; vascular endothelial growth factor; interleukin-6

Introduction

Branch retinal vein occlusion (BRVO) is a common retinal vascular disease and often results in macular oedema, which is the most frequent cause of visual impairment in patients with BRVO.[1,2] The expression of many cytokines is increased in RVO and cytokine levels are elevated in the ocular fluid of patients with RVO.[3–5] Thus, to assess the severity of macular oedema with BRVO by obtaining a sample of the aqueous humour or vitreous fluid at operation is of critical importance.[4] However, surgical harvesting of vitreous fluid is associated with the risk of vitreous haemorrhage, retinal tears, and retinal detachment, whereas it is difficult to obtain vitreous samples for diagnostic or investigative purposes without performing surgery. On the other hand, obtaining aqueous samples is a far easier and less risky procedure. If the cytokine levels in aqueous humour reflect those in vitreous fluid, we could investigate the pathogenesis and severity of macular oedema with BRVO by measuring cytokines in aqueous samples. However, it has been unclear whether the aqueous levels of substances, such as vascular endothelial growth factor (VEGF) and interleukin-6 (IL-6), are related to their vitreous levels. Additionally, little is known about the relationship between cytokines in the vitreous

fluid and those in the aqueous humour in macular oedema with BRVO.

Recently, numerous cytokines produced in the eye have been suggested to play a role in the pathogenesis and progression of diabetic macular oedema (DMO).[6–9] VEGF causes conformational alterations, such as phosphorylation and changes in protein content in the tight junctions of retinal vascular endothelial cells,[10,11] which play a role in the increase in vascular permeability.[12] The expression of VEGF is induced by hypoxia in retinal cells[13] and indirectly by IL-6.[14] IL-6 is a multifunctional cytokine that has the capacity to increase endothelial permeability through its induction of gap junction formation between adjacent cells as a result of the rearrangement of actin filaments.[15] As we reported previously, the aqueous levels of both VEGF and IL-6 are elevated in patients with macular oedema in BRVO and the aqueous level of VEGF was correlated with the severity of the macular oedema with BRVO, suggesting that these cytokines may contribute to the pathogenesis of macular oedema with BRVO.[16] Taken together, these findings suggest that these cytokines in the vitreous fluid and aqueous humour may show some relationship. Therefore, in this study, we investigated whether the VEGF and IL-6 levels in aqueous humour were related to those in vitreous fluid and to the severity of macular oedema with BRVO.

Materials and methods

Undiluted aqueous samples and undiluted vitreous samples were harvested at the start of combined vitrectomy and cataract operation after 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 Helsinki Declaration of 1975 (the 1983 revision), and the institutional review board also approved the protocol for the collection of aqueous humour, vitreous fluid, and blood samples at the Hiroshima University School of Medicine and Hiroshima Prefectural Hospital. Both aqueous samples and vitreous samples were obtained from 24 BRVO patients that matched the indication of combined vitrectomy and cataract operation. The indication of combined vitrectomy and cataract operation were as follows: (1) clinically detectable diffuse macular oedema or cystoid macular oedema of more than 1 month duration before vitrectomy, (2) best-corrected visual acuity worse than 20/40 before combined vitrectomy and cataract operation, and (3) prolonged macular oedema even after photocoagulation. The inclusion criteria for this study were cases of macular oedema with BRVO for which a combined vitrectomy and cataract operation was performed. Significant macular oedema was defined as retinal thickening of one optic disc area or greater in size, involving the fovea.[17] The exclusion criteria for this study were as follows: (1) previous ocular surgery, (2) patients with diabetes mellitus and diabetic retinopathy, (3) patients with iris rubeosis, (4) a history of ocular inflammation and vitreoretinal disease, and (5) patients who had the complications, such as intraoperative capsule breaks and dialysis during cataract surgery and long duration (over 20 min) of cataract surgery to avoid the possibility of influence of the vitreous levels of VEGF and IL-6. Combined vitrectomy and cataract operation was performed at the Hiroshima University School of Medicine and Hiroshima Prefectural Hospital.

Preoperative and operative fundus findings were recorded for each subject. The fundus findings were preoperatively confirmed by standardised fundus colour photography and fluorescein angiography, and a preset lens with a slit-lamp. A masked grader independently assessed the ischaemic occlusion of BRVO from photographs. Fundus photographs were taken with a Topcon 50° digital fundus camera, and panoramic images were made using Photoshop (Adobe Systems Inc., San Jose, CA, USA) and saved in BMP format. The panoramic images were then analysed using the public domain Scion Image program developed at Scion Corporation and available on the Internet at http://www.scioncorp.com/.[18] For digital fundus photography, the disc area was circumscribed using a cursor and then measured, as was also the case for the nonperfused area. The area of retinal photocoagulation was excluded when calculating the size of the nonperfused area. Also, the nonperfused area divided by the disc area was defined as the degree of retinal ischaemia.

The retinal thickness of the central fovea was measured by optical coherence tomography (OCT) (Zeiss–Humphrey Ophthalmic Systems, Dublin, CA, USA).[19] The fundi were scanned with a measurement beam focused on the horizontal and vertical planes crossing the central fovea, which was determined by the fundus photograph. All eyes were examined at scan lengths of 2.8 and 5.0 mm. The retinal thickness of the central fovea was defined as the length between the inner limiting membrane and the retinal pigment epithelium. It was automatically measured by computer. The severity of macular oedema was graded by the OCT-measured retinal thickness.

Samples of aqueous humour (100–200 μl) and vitreous fluid (300–500 μl) were collected into sterile tubes at the time of combined surgery and were rapidly frozen at -80°. Blood samples were simultaneously collected and centrifuged at 3000 *g* for 5 min to obtain plasma, and then aliquoted and stored at -80° until they were assayed.

VEGF and IL-6 were measured in the aqueous and vitreous samples from all eyes as well as in the plasma samples. The concentrations of VEGF and IL-6 were measured by enzyme-linked immunosorbent assay using human VEGF and IL-6 immunoassays (R&D Systems, Minneapolis, MN, USA) according to the manufacturer's instructions, and the details have been reported previously.[6,8,16] The VEGF kit used permitted the detection of two of the four VEGF isoforms, $VEGF_{121}$ and $VEGF_{165}$. The levels of these factors in the aqueous and vitreous samples and plasma were within the detection range of the assays, with the minimum detectable concentration being 15.6 pg/ml for VEGF (intra-assay coefficient of variation (CV): 5.3% and interassay CV: 6.5%) and 0.156 pg/ml for IL-6 (intra-assay CV: 5.4% and interassay CV: 6.8%).

Analyses were performed with SAS System software (ver. 9.1; SAS Institute Inc., Cary, NC, USA). Results are presented as the mean (SD). To examine correlations, Spearman's rank–order correlation coefficients were calculated, and the correlations were graphically represented by regression line. Two-tailed *P*-values of less than 0.05 indicated statistical significance.

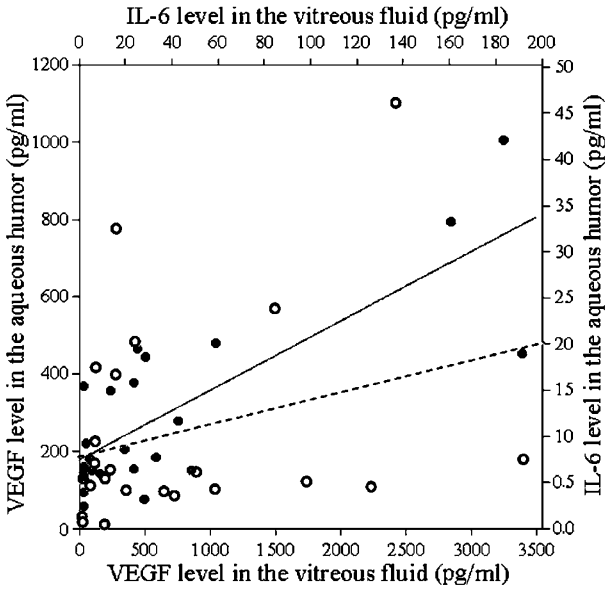

Figure 1 The aqueous humour level of VEGF was significantly correlated with the vitreous fluid level of VEGF ($\rho = 0.7625$, $P < 0.0001$). The aqueous level of IL-6 was not significantly correlated with the vitreous level of IL-6 ($\rho = 0.2930$, $P = 0.1429$). Filled circle, levels of VEGF; open circle, level of IL-6; underline, VEGF regression; and dotted line, IL-6 regression.

Results

Twenty-four patients with BRVO fulfiled the entry criteria. The male/female ratio was 8/16, and their ages ranged from 40 to 80 years (64.5 ± 10.2). The duration of BRVO ranged from 1 month to 10 months, with an average of 3.6 ± 2.5 months. Before surgery, photocoagulation had been performed in 12 eyes (mean: 288 shots; range 64–919 shots). The OCT-measured average preoperative retinal thickness was $509 \pm 148\ \mu$m (range 316–854 μm).

The aqueous level of VEGF (299.1 pg/ml (62.4–1010)) was significantly correlated with the vitreous level of VEGF (671.6 pg/ml (31.2–3380)) ($\rho = 0.7859$, $P < 0.0001$) (Figure 1). The aqueous level of IL-6 (10.1 pg/ml (0.6–46.3)) was not significantly correlated with the vitreous level of IL-6 (40.1 pg/ml (0.945–192)) ($\rho = 0.2965$, $P = 0.1594$) (Figure 1).

The aqueous levels of VEGF and IL-6 were significantly higher than the plasma levels (115.0 pg/ml (15.6–446), $P < 0.0001$; 6.47 pg/ml (0.15–139), $P < 0.0001$, respectively). The vitreous levels of VEGF and IL-6 were significantly higher than their plasma levels ($P = 0.0002$, $P < 0.0001$, respectively). However, no correlation was observed between the aqueous or vitreous and plasma levels of these cytokines (VEGF, $\rho = 0.1130$, $P = 0.5877$; $\rho = 0.0791$, $P = 0.7043$; IL-6, $\rho = 0.0604$, $P = 0.7719$; $\rho = 0.0061$, $P = 0.9767$; respectively). These data suggest that the VEGF and IL-6 levels in the aqueous humour and vitreous fluid were not elevated through the breakdown of the BRB and/or ocular blood.

Vitreous levels of VEGF and IL-6 were significantly correlated with the nonperfusion area of BRVO ($\rho = 0.8698$, $P < 0.0001$ and $\rho = 0.5435$, $P = 0.0061$, respectively) (Figure 2a). Aqueous levels of VEGF and IL-6 were also significantly correlated with the nonperfusion area of BRVO ($\rho = 0.7246$, $P < 0.0001$ and $\rho = 0.4517$, $P = 0.0267$, respectively) (Figure 2b). Furthermore, the vitreous levels of VEGF and IL-6 and aqueous level of VEGF were significantly correlated with the severity of macular oedema of BRVO ($\rho = 0.6998$, $P = 0.0001$; $\rho = 0.4362$, $P = 0.0331$; $\rho = 0.4505$, $P = 0.0272$, respectively) (Figure 3a and b), but the aqueous level of IL-6 was not significantly correlated with the severity of macular oedema ($\rho = 0.2708$, $P = 0.2005$) (Figure 3a and b).

Discussion

Recently, many effective approaches have been reported to the treatment of macular oedema with BRVO, such as photocoagulation, triamcinolone injection, and vitrectomy.[20–22] The absence of posterior vitreous detachment (PVD) can contribute to the occurrence of persistent macular oedema in retinal vascular occlusion.[19,23] Saika *et al*[22] reported on the effectiveness of vitrectomy combined with surgical PVD for macular oedema associated with BRVO. In this study, the patients

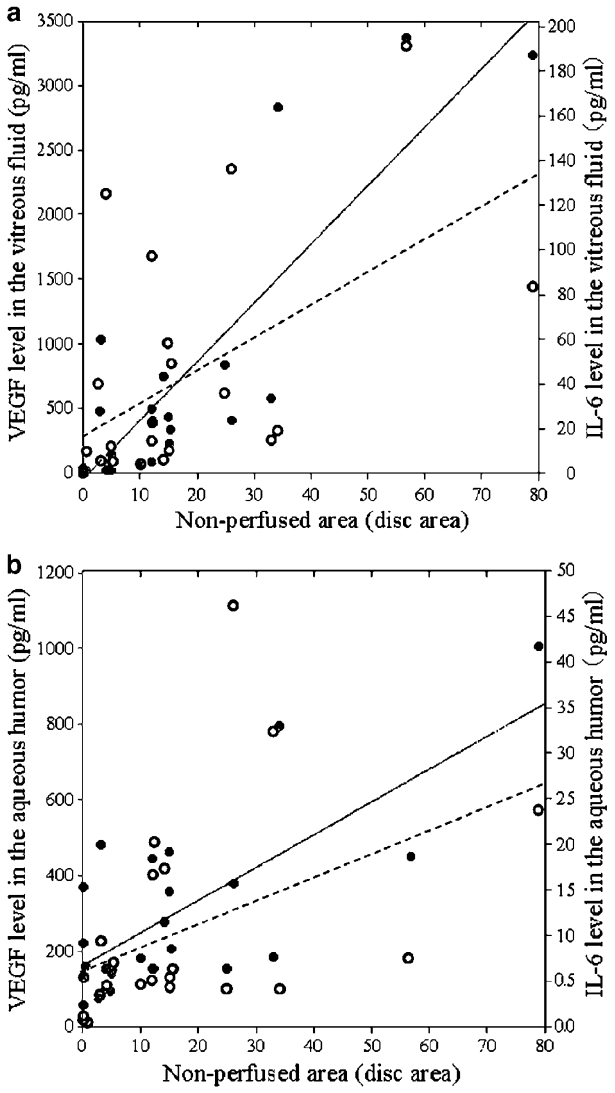

Figure 2 (a) Correlation between the nonperfusion area of BRVO and the vitreous levels of VEGF and IL-6. Vitreous levels of VEGF and IL-6 were significantly correlated with the nonperfusion area of BRVO ($\rho = 0.8708$, $P < 0.0001$; $\rho = 0.6332$, $P = 0.0016$, respectively). Filled circle, vitreous levels of VEGF; open circle, vitreous level of IL-6; underline, VEGF regression; and dotted line, IL-6 regression. (b) Correlation between the nonperfusion area of BRVO and the aqueous levels of VEGF and IL-6. Aqueous levels of VEGF and IL-6 were also significantly correlated with the nonperfusion area of BRVO ($\rho = 0.7344$, $P < 0.0001$; $\rho = 0.4841$, $P = 0.0157$, respectively). Filled circle, aqueous levels of VEGF; open circle, aqueous level of IL-6; underline, VEGF regression; and dotted line, IL-6 regression.

with macular oedema in BRVO were relatively aged (average 64.5 years old), therefore they were already suffering from cataracts. Thus, we performed combined vitrectomy and cataract operation for macular oedema associated with BRVO. The complications, such as intraoperative capsule breaks and dialysis during

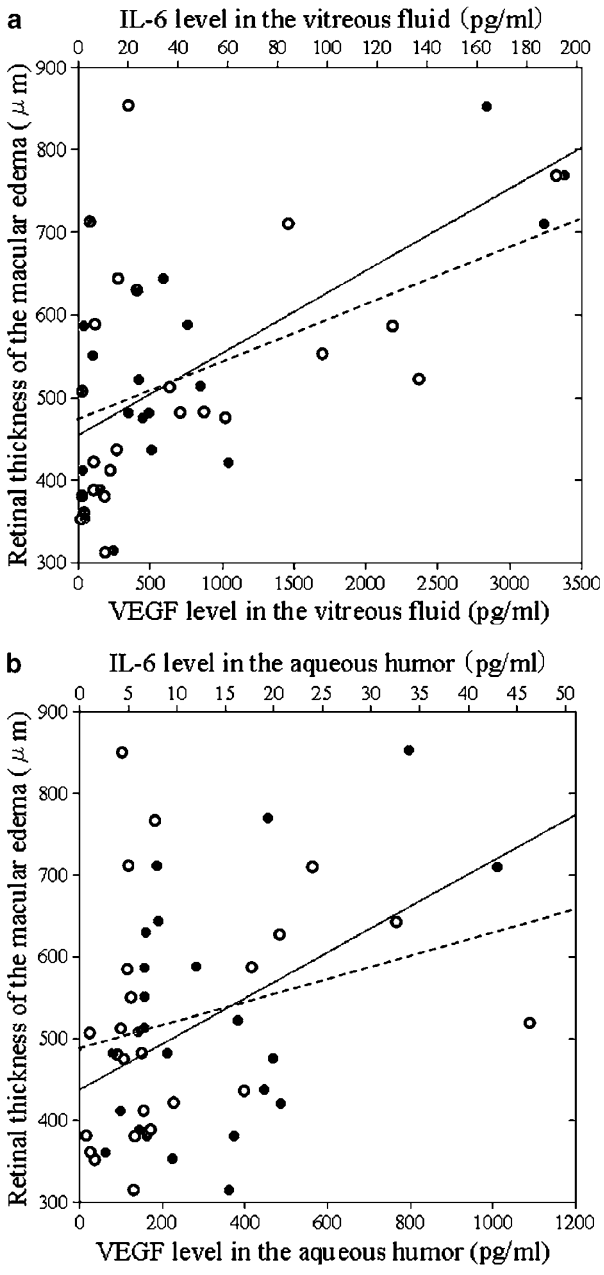

Figure 3 (a) Correlation between the severity of macular oedema of BRVO and the vitreous levels of VEGF and IL-6. The vitreous levels of VEGF and IL-6 were significantly correlated with the severity of macular oedema of BRVO ($\rho = 0.6523$, $P = 0.0003$; $\rho = 0.4675$, $P = 0.0194$, respectively). Filled circle, vitreous levels of VEGF; open circle, vitreous level of IL-6; underline, VEGF regression; and dotted line, IL-6 regression. (b) Correlation between the severity of macular oedema of BRVO and the aqueous levels of VEGF and IL-6. The aqueous level of VEGF was significantly correlated with the severity of macular oedema of BRVO ($\rho = 0.4333$, $P = 0.0270$), but the aqueous level of IL-6 was not significantly correlated with the severity of macular oedema ($\rho = 0.1701$, $P = 0.3955$). Filled circle, aqueous levels of VEGF; open circle, aqueous level of IL-6; underline, VEGF regression; and dotted line, IL-6 regression.

cataract surgery or a long duration of cataract surgery, may possibly affect the vitreous levels of VEGF and IL-6. However, in this study, there were no cases with intraoperative capsule breaks and dialysis during cataract surgery or a long duration of cataract surgery. Thus, the invasiveness of cataract surgery should not have an effect on the data.

In this study, we collected the aqueous and vitreous samples from the same patients at their operations and measured the levels of VEGF and IL-6 in patients with BRVO. We hypothesised that the aqueous levels of both VEGF and IL-6 might be correlated with their vitreous levels and with the severity of the ischaemic condition with BRVO. Our present findings and previous results showed that not only the vitreous levels of VEGF and IL-6 but also the aqueous levels of these two substances were correlated with the severity of the ischaemic condition with BRVO.[4,16] VEGF and IL-6 are known to be upregulated in retinal glial cells during retinal hypoxia.[24] It has been reported that a vitreous-to-aqueous gradient promotes the anterior diffusion of VEGF, potentially accounting for the occurrence of anterior-segment neovascularisation in conjunction with retinal ischaemia.[4,7] However, it has been unclear whether the aqueous levels of VEGF and IL-6 reflect their vitreous levels. In the present study, we simultaneously measured the cytokine levels in the aqueous humour and vitreous fluid.

We found that the aqueous and vitreous levels of VEGF were correlated with each other and that the average vitreous level of VEGF was significantly higher than the average aqueous level. However, the individual data showed that the VEGF level was a little higher in the aqueous humour than in the vitreous fluid in nine cases. The vitreous level of VEGF was higher than its aqueous level by 678 pg/ml in 15 cases. Meanwhile, in nine cases, the VEGF level was lower in the vitreous than in the aqueous; however, the difference was only 137 pg/ml. VEGF consists of four different isoforms.[25–27] They differ in their localisation, probably as a result of differential affinities for heparan sulphate proteoglycans that are found on the cell surface.[25,28] In this study, we simultaneously measured two isoforms of VEGF ($VEGF_{121}$ and $VEGF_{165}$) using the VEGF kit. These findings possibly suggest that the difference in the VEGF concentration between aqueous humour and vitreous fluid may depend on the localisation of these VEGF isoforms. While the factors associated with the expression of these isoforms are not clear, further investigation will be needed to confirm this. Besides, it has been reported that the blood–aqueous barrier function can be destroyed in BRVO.[29,30] Thus, the leakage of VEGF from the vessel may lead secondarily to the aqueous increased VEGF.

Unlike VEGF, the aqueous level of IL-6 was not significantly correlated with the vitreous level of IL-6, although the aqueous level and vitreous level of IL-6 was significantly higher than the plasma level. It has been shown that a wide range of ocular tissues can produce IL-6 *in vitro* and *in vivo*, such as corneal epithelial cells and keratocytes,[31] iris and cilliary body explants,[32] cytokine-stimulated human pigment epithelial cells,[33,34] ischaemic retina,[35] and hypoxia-induced or cytokine-stimulated vascular endothelial cells, and vascular smooth muscle cells.[36] Inflammatory cells, such as mast cells and macrophages, are known to be able to stimulate IL-6 secretion from leukocytes and human vascular endothelial cells in ischaemic and inflammatory conditions.[37–39] Thus, the reason that the IL-6 level was higher in the aqueous humour and vitreous fluid than that in the plasma level is possibly because IL-6 in the aqueous humour and vitreous fluid comes from intraocular sources, such as ocular and inflammatory cells. Also, Cohen *et al*[14] suggested that IL-6 may induce angiogenesis indirectly by stimulating VEGF expression. Therefore, there is the possibility that IL-6 in the aqueous humour and vitreous fluid may be induced by intraocular sources, according to the increasing VEGF expression.

The vitreous levels of VEGF and IL-6 and the aqueous level of VEGF were significantly correlated with the severity of macular oedema of BRVO. The expression of VEGF is induced by hypoxia in retinal cells.[13] In addition, VEGF causes rearrangement of actin filaments and increases endothelial permeability by promoting the phosphorylation of the tight junction proteins ZO-1 and occludin.[10,11] These findings suggest that in patients with BRVO, vascular occlusion induces the expression of VEGF, resulting in blood–retinal barrier breakdown and increased vascular permeability. Indeed, not only the vitreous levels of VEGF and IL-6 but also the aqueous levels of these two substances were correlated with the nonperfusion area of BRVO. Thus, VEGF may contribute to the development and progression of vasogenic macular oedema in BRVO. However, the aqueous levels of IL-6 were not significantly correlated with the severity of macular oedema, although they were significantly correlated with the size of the nonperfused area of BRVO and with the aqueous levels of VEGF. This is possibly because (1) the concentration of IL-6 was so much lower than that of VEGF in the aqueous humour, which does not induce macular oedema, although IL-6 indirectly induces VEGF expression,[14] and (2) the aqueous level of IL-6 was not correlated to the vitreous levels, as we described in this report. The results of this study suggest that BRVO-associated macular oedema is more greatly influenced by VEGF than by IL-6 in the aqueous humour, although we previously reported that aqueous and

vitreous levels of VEGF and IL-6 are correlated with the severity of DMO and increased vascular permeability in patients with DMO.[6,8] These data possibly suggest that increased expressions of VEGF and IL-6 in BRVO would have different roles from those two substances in diabetic retinopathy in the pathogenesis of macular oedema.

In conclusion, we found that the aqueous level of VEGF was significantly correlated with its vitreous level. In addition, the aqueous level of VEGF was significantly correlated with the nonperfusion area of BRVO and with the severity of the macular oedema in BRVO. These findings suggest that the aqueous level of VEGF may reflect its vitreous level, so that measurement of the aqueous level of VEGF may be clinically useful to indicate the severity of macular oedema with BRVO.

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