20122043A

別添１

研究報告書表紙

厚生労働科学研究費補助金

障害者対策総合研究事業

エピジェネティクス解析に基づいた網膜硝子体疾患に対する病態解明と発症予防および治療法の開発

平成２４年度　総括研究報告書

研究代表者　三村　達哉

平成２５（２０１３）年　４月

別添２

研究報告書目次

目　　次

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Ⅰ．総括研究報告

厚生労働科学研究費補助金（再生医療実用化研究事業）
（総括）研究報告書

エピジェネティクス解析に基づいた網膜硝子体疾患に対する病態解明と発症予防および治療法の開発

研究代表者　三村達哉　東京女子医科大学東医療センター眼科学教室　講師

研究要旨
エピジェネティクスとは、DNA の塩基配列の変化なしに、遺伝的しかも可逆的に遺伝子機能の発現が変化する現象で、DNA メチル化やヒストン修飾が関与する。癌をはじめあらゆる疾患におけるエピジェネティクス異常が、病態に関与し、診断や治療の標的となることが明らかになりつつあるが、感覚器疾患との関わりについてはまったく知られていない。本研究では、眼感覚器疾患の中で視機能に直接に影響を与える網膜硝子体疾患とエピジェネティクス異常の関係に焦点をあてて研究を行う。眼内での DNA、ヒストン、クロマチンのメチル化異常を調べることにより、原因不明であった眼疾患の病態を明らかにするとともに、特定の部位のメチレイションを抑制することにより、疾患の予防および、治療をすることを最終目標とする。

A．研究目的：

本研究では、眼内組織の老化のメカニズムを明らかにするために、近年 DNA の塩基配列に変化なしに遺伝的しかも可逆的に遺伝子機能に変化を及ぼすことが明らかになったエピジェネティクスの観点から、網膜硝子体疾患とエピジェネティクス異常の関係を調べることを目的とする。特定部位のエピジェンティクス変化（メチレイション）を抑制することにより、疾患の予防および、治療をすることを最終目標とする。

B．研究方法：

研究計画および方法

本研究は眼感覚器の中心となる網膜視機能障害に焦点をあて、網膜硝子体疾患における各種サイトカインとエピジェネティクスとの関係を調べることを目的としている。研究の対象は糖尿病網膜症、網膜静脈閉塞症、加齢性黄斑変性症に伴う黄斑浮腫などに対し手術を施行した患者である。硝子体手術を必要とする患者に同意を得たのち、前房水、硝子体液、血液、内境界膜を採取する。硝子体手術を必要とした黄斑円孔および黄斑上膜の症例をコントロールとする。

Ⅰ 研究デザイン

①症例対照研究、②前向きコホート研究、対象：黄斑浮腫患者、非黄斑浮腫患者（黄斑上膜、黄斑円孔）、症例数：criteria を満たす年間手術数は約 160 例で、そのうち年間 100 例を目標に研究にエントリーする。

（平成 23 年-24 年度）研究開始 2 年間は患者のエントリーならびに術前術後の網膜視機能の評価、サンプルの採取を行う。以下に示すように視力検査、細隙灯顕微鏡検査、眼底検査、蛍光眼底造影、光干渉断層計、網膜感度検査および網膜電図などを行い、各種網脈絡膜疾患に伴う網膜視機能評価を行う。

（平成 24-25 年度）

サンプルの採取およびサイトカイン濃度の測定

①症例対照研究黄斑浮腫患者群と非黄斑浮腫患者群との間における前房水、硝子体・血液中サイトカイン濃度、黄斑部血流速度の比較、組織学的研究、眼内液サイトカイン濃度およびと黄斑部血流速度との相関解析。測定候補サイトカインは VEGF, VEGF receptor-2 (VEGFR-2), Erythropoietin (EPO), ICAM-1, IL-6, PEDF , Vascular endothelial adhesion molecule-1 (VCAM-1) , Monocyte chmoattractant protein-1 (MCP-1), Stromal-derived factor-1 (SDF-1)。

②前向きコホート研究治療介入（硝子体手術単独、抗 VEGF 抗体硝子体注射、TA 硝子体注射）症例において、視力および黄斑部網膜厚を primary endpoint として、黄斑部血流速度変化、眼内液サイトカイン濃度変化との相関解析。エピジェネティクスの関係は次年度に行う。

（倫理面への配慮）

すべての研究は虎の門病院、東京大学、東京女子医科大学の倫理委員会の承認を得て行う。治療開発を前提とした研究であり、動物実験、臨床試験を行う予定のため、倫理委員会の指針、動物実験の対する指針、および研究に関与するあらゆる倫理指針を遵守する。動物の取り扱いは、苦痛を伴うものは必ず全身麻酔下に行い、両眼が失われる可能性のある場合は片眼のみに処置を行う。全ての実験において動物は the Association for Research in Vision and Ophthalmology の規約および、実験動物の飼養及び保管等に関する基準(総理府)に従って扱う。人を扱う研究では，ヘルシンキ宣言（世界医師会総会 World Medical Assembly)の勧告に従って行う。また遺伝子解析はヒトゲノム・遺伝子解析研究に関する倫理指針（文部科学省、厚生労働省、経済産業省）を遵守する。具体的には以下のように行う。患者を対象とする臨床試験においては十分な説明をした後、文書による同意を得てから行う（インフォームド・コンセント）。

C．研究結果：

本年度は網膜硝子体疾患の患者の硝子体手術時に硝子体サンプルを採取し保存したサンプルを用いて、VEGF 濃度、IL-6 濃度、VEGF-R2 を測定し、それぞれ網膜浮腫の重症度との正の相関、ならびに手術後の視力予後に影響しうることを証明した。また、硝子体サンプルを用いて、疾患発症に関与する特異的メチル化部位を特定するために全遺伝子配列を次世代シークエンサーにより調べており、次年度に得られたデータを解析する予定である。

D．考察：

硝子体サンプル中のサイトカイン濃度と術後の視力や網膜浮腫の重症度は相関することから、治療や硝子体手術術後の予後予測に硝子体サンプル中の液性因子濃度測定が有用であると考えられた。

E．結論

眼内硝子体サンプル中のサイトカイン濃度を測定することにより、術後や治療効果の判定ならびに、今後の予後予測にも有用であると考えられる。本年度から次年度への継続研究として、眼内硝子体サンプル中の、エピジェネティクス変化ならびに眼内老化との関わりについて、現在研究を行っている。

F．健康危険情報

本研究の結果により、健康に及ぼす危険事項は確認できなかった。

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H. 知的財産権の出願・登録状況

1. 特許取得　なし
2. 実用新案登録　なし
3. その他・賞罰
   平成２４年４月　三村達哉　第 17 回 ROHTO AWARD

別添５

研究成果の刊行に関する一覧表

書籍

著者氏名	論文タイトル名	書籍全体の編集者名	書　籍　名	出版社名	出版地	出版年	ページ
なし							

雑誌

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Mimura T, Obata H, Usui T, Mori M, Yamagami S, Funatsu H, Noma H, Amano S.	Pinguecula and diabetes mellitus.	Cornea	31	264-268	2012
Noma H, Funatsu H, **Mimura T**, Tatsugawa M, Shimada K, Eguchi S.	Vitreous Inflammatory Factors and Serous Macular Detachment in Branch Retinal Vein Occlusion.	Retin	32	86-91	2012
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Mimura T, Nakashizuka T, Kami J, Kohmura M, Sato S, Dou K, Mori M.	Bilateral choroidal detachment after cord blood stem cell transplantation in an adult patient with acute myeloid leukemia.	Int Ophthalmol	32	379-381	2012
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Mimura T, Noma H, Funatsu H.	Development of surgical treatment for vitreous disease.	Surgical Science	3	507-551	2012
Mimura T, Yokoo S, Yamagami S.	Tissue engineering of corneal endothelium.	J Funct Biomater	3	726-744	2012
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Mimura T, Yamagami S, Amano S.	Mimura T, Yamagami S, Amano S. Corneal Endothelial Regeneration and Tissue Engineering	Prog Retin Eye Res	Jan 23.	In press	2013
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Mimura T, Yamagami S, Kamei Y, Goto M, Kondo A, Matsubara M.	Measurement of Specific Tear IgE With ImmunoCAP Rapid. Journal of Investigational Allergology and Clinical Immunology in press.	J Investig Allergol Clin Immunol		In press	
Mimura T, Noma H, Funatsu H.	Epigenetic regulation of retinal stem cells.	International Journal of Stem Cell Research and Transplantation		In press	
Mimura T, Yamagami S, Kamei Y, Goto M, Matsubara M.	Influence of axial length on conjunctivochalasis.	Cornea		In press	
Noma H, Funatsu H, Shimada K, **Mimura T**.	Influence of pars plana vitrectomy on macular sensitivity and morphology in patients with branch retinal vein occlusion and serous retinal detachment.	Ophthalmic Surg Lasers Imaging Retina	44	160-167	2013
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代表論文の1報のみ添付する

CLINICAL SCIENCES

Association of Inflammatory Factors With Macular Edema in Branch Retinal Vein Occlusion

Hidetaka Noma, MD; Tatsuya Mimura, MD; Shuichiro Eguchi, MD

Objective: To evaluate the association between vitreous fluid levels of inflammatory factors and macular edema in patients with branch retinal vein occlusion (BRVO).

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Association of Inflammatory Factors With Macular Edema in Branch Retinal Vein Occlusion

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BRANCH RETINAL VEIN OCCLUSION (BRVO) often results in macular edema, which is the chief cause of visual impairment in patients with BRVO. Although the pathogenesis of macular edema in these patients is unclear, retinal changes due to BRVO (including hemorrhage) are known to cause local inflammation. After retinal vein occlusion, there is increased rolling and adhesion of leukocytes to the retinal vein walls that lead to stagnation of blood flow,[1] so inflammation may play a key role in the pathogenesis of BRVO. The role of inflammation is supported by reports that intravitreal injection of triamcinolone acetonide lessens macular edema in patients with BRVO[2] and that the aqueous flare value is significantly higher in patients with retinal vein occlusion than in healthy individuals.[3]

Various molecules that are secreted into the vitreous fluid may be associated with ocular abnormalities, although the vitreous levels of soluble inflammatory factors might not necessarily reflect their tissue levels, especially the amounts in the retinal microenvironment. However, the concentrations of soluble factors secreted into the vitreous fluid have been reported[4] to influence visual prognosis. There is evidence that upregulation of inflammatory factors, including vascular endothelial growth factor (VEGF), VEGF receptor 2 (VEGFR-2), intercellular adhesion molecule 1 (ICAM-1), interleukin 6 (IL-6), and monocyte chemotactic protein 1 (MCP-1), or downregulation of anti-inflammatory factors, such as pigment epithelium-derived factor (PEDF), and a subsequent increase in leukocyte-endothelial interactions contribute to breakdown of the blood-retinal barrier (BRB).[5-7] The levels of VEGF, IL-6, soluble ICAM-1 (sICAM-1), soluble VEGFR-2 (sVEGFR-2), and PEDF in the vitreous fluid are independently related to vascular permeability in patients with BRVO and macular edema.[8-10] Blocking the actions of inflammatory factors has been shown[5] to prevent leukostasis and an increase in retinal vascular permeability in rats, and development of macular edema in patients with BRVO has been reported[7,11] to be accompanied by elevation of cytokines that regulate the inflammatory response. Thus, various inflammatory cytokines and other factors influence vascular permeability in the eye and are associated with macular edema in patients with BRVO.

Recently, long pentraxin 3 (PTX3) was reported to be an early indicator of myocardial infarction[12] and a predictor of

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3-month mortality after acute myocardial events.[13] Long pentraxin 3 is an acute-phase protein that is involved in innate immunity and inflammation. Pentraxins are a family of acute response proteins comprising 3 members—C-reactive protein, serum amyloid P, and PTX3—and these proteins are classic acute-phase reactants that closely reflect the level of inflammatory activity.[14,15] Long pentraxin 3 is induced by cytokines and is produced mainly by vascular endothelial cells, fibroblasts, and cells in some other extrahepatic tissues,[14,16-20] unlike the other 2 family members that are synthesized primarily in the liver.[21,22] In an animal model, there was a rapid increase in PTX3 expression after reperfusion of the ischemic superior mesenteric artery territory.[23] More important, overexpression of PTX3 was accompanied by an increase in death and tissue damage after intestinal ischemia and reperfusion.[23] In addition, PTX3 increases vascular permeability.[24] These findings suggest that PTX3 may play an important role in the pathogenesis of macular edema associated with BRVO. However, the level of PTX3 expression in patients with BRVO and its relationship to the pathogenesis of macular edema are unclear, just as the relative contribution of each of the molecules evaluated herein to the development of macular edema remains uncertain. Accordingly, we measured the vitreous fluid levels of 6 inflammatory factors (including PTX3) and 1 anti-inflammatory factor in patients with BRVO and macular edema, focusing on molecules that have been linked to the onset or exacerbation of this condition. The association between each of these molecules and the severity of macular edema was then assessed.

METHODS

PARTICIPANTS

Undiluted vitreous fluid samples were harvested at the start of pars plana vitrectomy (PPV) after written informed consent was obtained from each participant 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 (1983 revision). The institutional review boards of Tokyo Women's Medical University and Eguchi Eye Hospital approved the protocol for collection and testing of vitreous fluid and blood samples. This was a retrospective case-control study of 60 Japanese patients who underwent PPV in 1 eye (39 with BRVO and 21 with idiopathic macular hole [MH]) to treat macular edema. Seventy-two consecutive patients with BRVO who sought care at the hospitals associated with Tokyo Women's Medical University or Eguchi Eye Hospital between August 11, 2009, and November 15, 2011, were screened, using the criteria listed in the next sentence, and vitreous fluid samples were obtained from the 39 patients enrolled. The indications for PPV were (1) clinically detectable diffuse macular edema or cystoid macular edema persisting for more than 3 months and (2) best-corrected visual acuity worse than 20/40.

The Branch Vein Occlusion Study[25] demonstrated the effectiveness of argon laser photocoagulation for BRVO, but it was recommended that this should not be performed within 3 months of occurrence, during which time spontaneous improvement may occur. The absence of posterior vitreous detachment can contribute to persistent macular edema in patients with retinal vascular occlusion.[26] Saika et al[27] reported on the effectiveness of PPV combined with surgical posterior vitreous detachment for macular edema in patients with BRVO.

Table 1. Clinical and Laboratory Characteristics of the BRVO and MH Groups

Variable	BRVO Group	MH Group	*P* Value
No. of participants[a]	39	21	
Sex, No.			.66
Female	20	12	
Male	19	9	
Age, mean (SD), y	69.2 (9.6)	68.8 (8.4)	.88
Blood pressure, mean (SD), mm Hg			
Systolic	134 (14)	121 (11)	<.001
Diastolic	78 (8)	74 (8)	.07
Hypertension, No.	22	3	.002
Hyperlipidemia, No.	12	4	.33
Duration of BRVO, mean (SD), mo	5.1 (2.4)	...	...

Abbreviations: BRVO, branch retinal vein occlusion; ellipsis, not applicable; MH, macular hole.
[a] Number of participants with data.

It has also been reported[28,29] that PPV contributes to an increase in oxygen tension in the inner retina. If retinal oxygen tension increases after PPV, macular edema would be lessened for several reasons. First, an increase in oxygen tension would reduce VEGF production and thus decrease vascular permeability. Second, an increase in oxygen tension would alleviate autoregulatory arteriolar vasoconstriction and thus reduce the hydrostatic pressure in the retinal capillaries and venules. This would decrease water flux from the vascular compartment to the tissue compartment and reduce edema according to the Starling law. Finally, PPV reduces the intraocular levels of various other inflammatory factors in addition to VEGF,[30] and this may be another mechanism by which it alleviates macular edema in patients with BRVO. In fact, it has been reported[31,32] that PPV improves both functional and tomographic outcomes in patients with BRVO and macular edema. Accordingly, we performed PPV in patients with clinically detectable diffuse macular edema or cystoid macular edema more than 3 months after the onset of BRVO.

Thirty-three of the 72 patients were excluded because of previous ocular surgery or intravitreous injection of anti-VEGF agents or triamcinolone acetonide in 23 patients, diabetic retinopathy in 2 patients, previous retinal photocoagulation in 7 patients, and a history of ocular inflammation or vitreoretinal disease in 1 patient. Patients with intravitreous injection of anti-VEGF agents or triamcinolone acetonide were excluded because such treatment could influence vitreous fluid levels of inflammatory factors. Vitreous fluid samples were also obtained from 21 patients with nonischemic ocular diseases as a control group (MH group). None of the patients in the MH group had proliferative vitreoretinopathy. The mean (SD) age of the BRVO group (19 men and 20 women) was 69.2 (9.6) years, and the control group (9 men and 12 women) was aged 68.8 (8.4) years. The mean duration of BRVO was 5.1 (2.4) months (range, 3-11 months). Clinical and laboratory characteristics of the BRVO and MH groups are shown in **Table 1**.

FUNDUS FINDINGS

Both preoperative and operative fundus findings were recorded for each participant. A masked grader (H.N.) independently assessed ischemic retinal vascular occlusion by examining fluorescein angiograms. The ischemic region of the retina was measured with the public domain Scion Image program (Scion Corp), as reported previously.[8-10] On digital fundus pho-

Table 2. Vitreous Fluid Levels of Factors in the Groups

Variable	Median (Interquartile Range) BRVO Group	MH Group	P Value
sVEGFR-2, pg/mL	1500 (1083-2035)	1020 (721-1343)	.002
VEGF, pg/mL	229 (33.9-1353)	15.6 (15.6-31.2)	<.001
sICAM-1, ng/mL	8.20 (5.33-15.6)	4.50 (3.60-5.65)	<.001
IL-6, pg/mL	10.7 (5.53-29.0)	1.00 (0.50-1.18)	<.001
MCP-1, pg/L	1190 (747-1993)	458 (375-636)	<.001
PTX3, ng/mL	0.86 (0.50-1.62)	0.50 (0.50-0.81)	.01
PEDF, ng/mL	25.6 (8.14-40.7)	59.9 (25.0-101)	.005

Abbreviations: BRVO, branch retinal vein occlusion; IL-6, interleukin 6; MCP-1, monocyte chemotactic protein 1; MH, macular hole; PEDF, pigment epithelium-derived factor; PTX3, pentraxin 3; sICAM-1, soluble intercellular adhesion molecule 1; sVEGFR-2, soluble vascular endothelial growth factor (VEGF) receptor 2.

tographs, the disc area was outlined with a cursor and then measured, and the same was done for the nonperfused area. The severity of retinal ischemia was assessed as the nonperfused area divided by the disc area.

Optical coherence tomography was performed in each participant within 1 week before PPV (Zeiss-Humphrey Ophthalmic Systems). The thickness of the central fovea was defined as the distance between the inner limiting membrane and the retinal pigment epithelium (including any serous retinal detachment) and was automatically measured by computer software (Zeiss-Humphrey Ophthalmic System). The thickness of the neurosensory retina was defined as the distance between the inner and outer neurosensory retinal surfaces,[26] and the severity of macular edema was graded from the measured retinal thickness.

SAMPLE COLLECTION

Samples of undiluted vitreous fluid (0.5-1.0 mL) were collected at the start of PPV by aspiration into a 1-mL syringe attached to the vitreous cutter before the intravitreal infusion of balanced salt solution was begun. The vitreous samples were immediately transferred into sterile tubes and were rapidly frozen at −80°C. Blood samples were collected simultaneously and were centrifuged at 3000*g* for 5 minutes to obtain plasma, after which aliquots were stored at −80°C until assays were performed.

MEASUREMENT OF INFLAMMATORY AND ANTI-INFLAMMATORY FACTORS

The levels of VEGF, sVEGFR-2, sICAM-1, IL-6, MCP-1, and PTX3 were measured in vitreous samples from the same eye and in plasma samples by enzyme-linked immunosorbent assay, using kits for human VEGF, sVEGFR-2, IL-6, MCP-1, and PTX3 (R&D Systems); sICAM-1 (Bender Med Systems); and PTX3 (Perseus Proteomics Inc).[8,10,33] Similarly, levels of anti-inflammatory PEDF were measured in vitreous samples with a human PEDF sandwich enzyme-linked immunosorbent assay kit (Chemicon International).[9] The VEGF kit was able to detect 2 of the 4 VEGF isoforms ($VEGF_{121}$ and $VEGF_{165}$), probably because these 2 shorter isoforms are secreted and the 2 longer isoforms are cell associated. Each assay was performed according to the manufacturer's instructions.

Table 3. Correlation of Vitreous Factors With the Nonperfused Area and Retinal Thickness

Variable	Nonperfused Area r	Nonperfused Area P Value	Retinal Thickness r	Retinal Thickness P Value
sVEGFR-2	0.19	.25	0.36	.02
VEGF	0.77	<.001	0.47	.003
sICAM-1	0.36	.02	0.56	<.001
IL-6	0.46	.004	0.41	.01
MCP-1	0.52	.001	0.63	<.001
PTX3	0.37	.02	0.39	.02
PEDF	−0.39	.02	−0.36	.02

Abbreviations: See Table 2; *r*, correlation coefficient.

STATISTICAL ANALYSIS

Analyses were performed with commercial software (SAS, version 9.1; SAS Institute Inc). A *t* test was used to compare normally distributed unpaired continuous variables between the 2 groups, and the Mann-Whitney test was used for variables with a skewed distribution. The χ^2 test or Fisher exact test was used to compare discrete variables. Differences between the median plasma and vitreous levels were assessed with the Wilcoxon single rank test. To examine relationships among the variables, Spearman rank order correlation coefficients or Pearson correlation coefficients were calculated. Statistical significance was set at $P < .05$, with 2-tailed values.

RESULTS

The vitreous fluid concentration of sVEGFR-2 (median [interquartile range]) was significantly higher in the BRVO group (1500 pg/mL [1083-2035]) than in the MH group (1020 pg/mL [721-1343]; $P = .002$) (**Table 2**). The vitreous fluid concentration of VEGF was significantly higher in the BRVO group (229 pg/mL [33.9-1353]) compared with the MH group (15.6 pg/mL [15.6-31.2]; $P < .001$) (Table 2). Likewise, vitreous sICAM-1 levels were significantly higher in the BRVO group (8.20 ng/mL [5.33-15.6]) than in the MH group (4.50 ng/mL [3.60-5.65]; $P < .001$) (Table 2). Furthermore, the vitreous level of IL-6 was significantly higher in the BRVO group (10.7 pg/mL [5.53-29.0]) than in the MH group (1.00 pg/mL [0.50-1.18]; $P < .001$), as was the vitreous level of MCP-1 (1190 pg/mL [747-1993] vs 458 pg/mL [375-636]; $P < .001$) and the vitreous level of PTX3 (0.86 ng/mL [0.50-1.62] vs 0.50 ng/mL [0.50-0.81]; $P = .01$) (Table 2). In contrast, the vitreous fluid level of PEDF was significantly lower in the BRVO group (25.6 ng/mL [8.14-40.7]) than in the MH group (59.9 ng/mL [25.0-101]; $P = .005$) (Table 2).

Vitreous fluid levels of VEGF, sICAM-1, IL-6, MCP-1, and PTX3 were significantly correlated with the nonperfused area of the retina in the BRVO group ($r = 0.77$, $P < .001$; $r = 0.36$, $P = .02$; $r = 0.46$, $P = .004$; $r = 0.52$, $P = .001$; and $r = 0.37$, $P = .02$, respectively) (**Table 3**). Conversely, the vitreous fluid level of PEDF showed a significant negative correlation with the nonperfused area in the BRVO group ($r = -0.39$, $P = .02$) (Table 3). However, the vitreous fluid level of sVEGFR-2 was not significantly correlated with the nonperfused area in this group ($r = 0.19$, $P = .25$) (Table 3).

Table 4. Correlation Matrix for Vitreous Factors

	sVEGFR-2		VEGF		sICAM-1		IL-6		MCP-1		PTX3		PEDF	
Variable	*r*	*P* Value	*r*	*P* Value	*r*	*P* Value	*r*	*P* Value	*r*	*P* Value	*r*	*P* Value	*r*	*P* Value
sVEGFR-2			0.14	.38	0.76	<.001	0.63	<.001	0.69	<.001	0.66	<.001	−0.12	.44
VEGF					0.34	.03	0.41	.01	0.46	.004	0.23	.19	−0.33	.04
sICAM-1							0.63	<.001	0.66	<.001	0.64	<.001	0.03	.87
IL-6									0.70	<.001	0.65	<.001	−0.10	.52
MCP-1											0.53	<.001	−0.39	.02
PTX3													0.04	.82

Abbreviations: See Table 2; *r*, correlation coefficient.

Vitreous fluid levels of sVEGFR-2, VEGF, sICAM-1, IL-6, MCP-1, PTX3, and PEDF were significantly correlated with the retinal thickness at the central fovea according to simple linear regression analysis ($r = 0.36$, $P = .02$; $r = 0.47$, $P = .003$; $r = 0.56$, $P < .001$; $r = 0.41$, $P = .01$; $r = 0.63$, $P < .001$; $r = 0.39$, $P = .02$; and $r = -0.36$, $P = .02$, respectively) (Table 3).

In the BRVO group, there were significant correlations between the vitreous fluid level of sVEGFR-2 and the levels of sICAM-1, IL-6, MCP-1, and PTX3 ($r = 0.76$, $P < .001$; $r = 0.63$, $P < .001$; $r = 0.69$, $P < .001$; and $r = 0.66$, $P < .001$; respectively) (**Table 4**). There were also significant correlations between the vitreous fluid level of VEGF and the levels of sICAM-1, IL-6, MCP-1, and PEDF in the BRVO group ($r = 0.34$, $P = .03$; $r = 0.41$, $P = .01$; $r = 0.46$, $P = .004$; and $r = -0.33$, $P = .04$, respectively) (Table 4). Furthermore, there was a significant correlation between the vitreous fluid level of sICAM-1 and the levels of IL-6, MCP-1, and PTX3 ($r = 0.63$, $P < .001$; $r = 0.66$, $P < .001$; and $r = 0.64$, $P < .001$, respectively) (Table 4). Moreover, there was a significant correlation between the vitreous fluid level of IL-6 and the levels of MCP-1 and PTX3 ($r = 0.70$, $P < .001$; and $r = 0.65$, $P < .001$, respectively) (Table 4), as well as a significant correlation between MCP-1 and PTX3 or PEDF ($r = 0.53$, $P < .001$; and $r = -0.39$, $P = .02$, respectively) (Table 4). In contrast, there was no significant correlation between the vitreous levels of sVEGFR-2 and VEGF ($r = 0.14$, $P = .38$) or between the vitreous levels of VEGF and PTX3 in the BRVO group ($r = 0.23$, $P = .19$) (Table 4). There were also no significant correlations between the vitreous level of PEDF and the levels of sVEGFR-2, sICAM-1, IL-6, and PTX3 in the BRVO group ($r = -0.12$, $P = .44$; $r = 0.03$, $P = .87$; $r = -0.10$, $P = .52$; and $r = 0.04$, $P = .82$, respectively) (Table 4).

In the BRVO group, the vitreous fluid levels of VEGF, IL-6, and MCP-1 were significantly higher (all $P < .001$) than the plasma levels of these molecules (18.1 pg/mL [15.6-44.1], 0.59 pg/mL [0.35-0.98], and 142 pg/mL [117-167], respectively), whereas the vitreous levels of sVEGFR-2, sICAM-1, and PTX3 were significantly lower (all $P < .001$) than their plasma levels (6750 pg/mL [5895-8245], 423 ng/mL [332-508], and 3.66 ng/mL [2.66-5.11], respectively).

COMMENT

There were 3 main findings in this study. First, vitreous fluid levels of sVEGFR-2, VEGF, sICAM-1, IL-6, MCP-1, and PTX3 were significantly higher in patients with BRVO and macular edema than in controls with MH. Second, vitreous fluid levels of sVEGFR-2, VEGF, sICAM-1, IL-6, MCP-1, PTX3, and PEDF were also correlated with the retinal thickness at the central fovea. Finally, there were significant correlations among the vitreous fluid levels of sICAM-1, IL-6, MCP-1, PTX3, and sVEGFR-2 in the BRVO group, as well as among the vitreous levels of sICAM-1, IL-6, MCP-1, PEDF, and VEGF.

These findings suggest that not only VEGF but also VEGFR-2, ICAM-1, IL-6, MCP-1, and PTX3 may play important roles in the occurrence of macular edema associated with BRVO. Vascular endothelial growth factor has a potent influence on vascular permeability, and its production is upregulated by retinal hypoxia in patients with BRVO and macular edema.[8] Breakdown of the BRB and retinal vascular hyperpermeability are important pathophysiologic features of macular edema associated with BRVO, and there is evidence that inflammation is a key mediator of both endothelial cell damage and BRB breakdown.[5-7] Upregulation of inflammatory factors, including VEGF, VEGFR-2, ICAM-1, IL-6, and MCP-1, as well as increased rolling and adhesion of leukocytes, is observed before and during the increase in retinal permeability.[5-7] Leukocyte recruitment is modulated by PTX3 in inflammation,[34] so its upregulation could also lead to an increase in vascular permeability.[24] This possibility is supported by the report[24] that the response of vascular permeability is less marked in PTX3-deficient mice. Thus, interactions among the network of inflammatory factors evaluated here may enhance vascular permeability. Activation of ICAM-1 and the subsequent increase in leukocyte-endothelial adhesion may be essential for VEGF to induce vascular hyperpermeability[5] because blocking ICAM-1 activity almost completely prevents VEGF-induced leukostasis and BRB breakdown.[35] However, blocking VEGF activity in the diabetic retina markedly reduces the upregulation of ICAM-1 as well as the increase in leukocyte adhesion and BRB breakdown.[36] These findings suggest that VEGF is the key factor mediating the response to hypoxia in the retina.

Interestingly, we found a significant correlation between the vitreous fluid level of sVEGFR-2 and the levels of various inflammatory factors (sICAM-1, IL-6, MCP-1, and PTX3) in patients with BRVO and macular edema, but there was no significant correlation between the vitreous fluid levels of sVEGFR-2 and VEGF. Binding of VEGF to VEGFR-2 triggers a signaling cascade that results in tyro-

sine phosphorylation of phospholipase Cγ,[37-39] which in turn increases the intracellular levels of inositol 1,4,5-triphosphate and diacylglycerol. Inositol 1,4,5-triphosphate increases the intracellular calcium level by promoting efflux of calcium from the endoplasmic reticulum. This increase in intracellular calcium stimulates sphingosine kinase to produce sphingosine 1-phosphate,[40] which then activates protein kinase C (PKC). Activated phospholipase Cγ also activates PKC by increasing the level of diacylglycerol, and activated PKC is a strong activator of nuclear factor κ B (NF-κB).[41] There is ample evidence that NF-κB promotes the transcription of inflammatory factors (including ICAM-1, IL-6, and MCP-1).[42-47] Nuclear factor–κB is found in almost all cell types and is involved in cellular responses to stimuli such as stress, proinflammatory gene expression (including cytokines, adhesion molecules, and chemokines), free radicals, UV irradiation, and bacterial or viral antigens in addition to its central role in the immune response.[48-50] It has also been reported[51-54] that VEGF, via the VEGFR-2–PKC axis, induces the production of proinflammatory cytokines (including IL-6 and MCP-1) in endothelial cells. Thus, VEGF promotes the expression of inflammatory factor messenger RNAs (including ICAM-1, IL-6, and MCP-1), mainly through the activation of PKC and NF-κB, indicating that VEGF induces the expression of inflammatory proteins by vascular endothelial cells through binding to VEGFR-2. This is supported by reports[53,55,56] that a specific VEGFR-2 antagonist blocks VEGF-induced expression of inflammatory factors (including ICAM-1, IL-6, and MCP-1) and also blocks activation of NF-κB by VEGF. Expression of the PTX3 gene also requires the activation of NF-κB.[57] In addition, Souza et al[24] reported that NF-κB activation was significantly suppressed in PTX3-deficient mice. Taken together with our results, these reports suggest that the vitreous level of sVEGFR-2 influences various inflammatory factors (including ICAM-1, IL-6, MCP-1, and PTX3) in patients with BRVO and macular edema. On the other hand, the vitreous level of sVEGFR-2 may be regulated independently of VEGF, although the VEGF–VEGFR-2 signaling pathway is considered essential for controlling vascular permeability.[58,59] The VEGF is upregulated by hypoxia through hypoxia-inducible factor 1α,[60] which is another transcription factor that regulates genes responding to hypoxia.[61] Vascular endothelial growth factor may act via an independent pathway to promote the retinal changes that occur in BRVO; therefore, additional studies are required to identify the mechanism. Differences in the activation of various transcription factors may determine the severity of ocular ischemic and inflammatory changes.

Considering our results, as well as the balance between VEGF and inflammatory cytokines, we should select treatment with anti-VEGF agents (to reduce the level of free VEGF) or triamcinolone acetonide (with a broad spectrum of action, as appropriate). Because the aqueous level of VEGF is significantly correlated with the vitreous level of VEGF,[62] measuring the concentrations of various molecules in aqueous humor by enzyme-linked immunosorbent assay or multiplex bead analysis could help with the selection of treatment between anti-VEGF agents, triamcinolone acetonide, or combined therapy. In addition, upregulation of inflammatory factors may be dependent on VEGFR-2 because there were significant correlations between the vitreous fluid level of sVEGFR-2 and the vitreous levels of 4 inflammatory factors (sICAM-1, IL-6, MCP-1, and PTX3) in our patients with BRVO and macular edema. Accordingly, multiple inflammatory factors could be inhibited by an antibody targeting VEGFR-2, so it may be worth also considering anti–VEGFR-2 therapy to treat macular edema in this population. However, a prospective clinical trial would be required to investigate the efficacy of such therapy.

This study also had some other limitations. For example, it is unclear from our data whether elevated vitreous levels of cytokines and chemokines were related to increased retinal vascular permeability or local production in the retina, but the mechanism involved may be revealed by animal studies.

In the present study, the vitreous fluid levels of sVEGFR-2, VEGF, sICAM-1, IL-6, MCP-1, PTX3, and PEDF were strongly correlated with retinal vascular permeability and the severity of macular edema. The sVEGFR-2 level was significantly correlated with the levels of sICAM-1, IL-6, MCP-1, and PTX3 but not with the level of VEGF. These findings suggest the importance of investigating relationships among VEGF and the cytokine network and may contribute to understanding the mechanism of macular edema in patients with BRVO and developing new treatments.

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Author Contributions: Dr Noma had full access to all the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.
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