Int Ophthalmol (2021) 41:1845–1853
https://doi.org/10.1007/s10792-021-01746-9

ORIGINAL PAPER

Cytokines associated with hemorrhage in proliferative diabetic retinopathy

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Received: 20 August 2020 / Accepted: 6 February 2021 / Published online: 20 February 2021

Abstract

Purpose To investigate aqueous cytokine levels in association with hemorrhage in proliferative diabetic retinopathy (PDR) in patients with type 2 diabetes mellitus.

Methods Sixty-six eyes with treatment-naïve PDR, including 26 hemorrhagic and 40 nonhemorrhagic eyes were included in this institutional study. Aqueous humor levels of interleukin (IL)-1b, IL-6, IL-8, IL-10, monocyte chemoattractant protein (MCP)-1, tumor necrosis factor (TNF)-α, vascular endothelial growth factor (VEGF), and soluble VEGF receptor-1 were obtained by multiplex bead assay. Visual acuity and hemorrhage area measurements were obtained, and correlations between cytokine levels and hemorrhage were identified.

Results Levels of MCP-1, TNF-α, and VEGF were higher in hemorrhagic eyes (1506.77 vs. 2131.31 pg/mL, 0.43 vs. 0.63 pg/mL, and 103.96 vs. 206.96 pg/mL; $P = 0.050$, 0.022, and 0.027, respectively). The levels of IL-8, MCP-1, TNF-α, and VEGF showed positive correlation with visual acuity ($P = 0.019$, 0.015, 0.001, and 0.014, respectively). The hemorrhage area revealed positive correlation with TNF-α and VEGF levels ($P = 0.001$ and < 0.001, respectively).

Conclusion The presence and amount of hemorrhage in PDR were associated not only with VEGF concentration, but also with the levels of certain inflammatory cytokines, suggesting a role of both VEGF and inflammation in hemorrhagic eyes.

Keywords Proliferative diabetic retinopathy (PDR) · Hemorrhage · VEGF · MCP-1 · TNF-a

Introduction

Diabetic retinopathy (DR) is a leading cause of blindness in adults aged 20–74 years [1]. While diabetic macular edema (DME) is responsible for most of the visual loss experienced by patients with type 2 diabetes mellitus (DM), blindness from PDR is caused by angiogenesis and fibrosis in the vitreous cavity [2]. Earlier, the Diabetic Retinopathy Study defined vitreous hemorrhage, neovascularization (NV), NV at disk area (NVD), and severe angiogenesis as risk factors for severe vision loss [3]. The presence of NV is the hallmark of proliferative DR (PDR), which progresses to a fibrotic phase with fibrovascular contraction, causing hemorrhages followed by fibrous membrane proliferation and tractional retinal detachment. About 68% of vitreous hemorrhage cases

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progress to visual acuity 5/200 or worse if untreated [4].

Upregulation of angiogenic cytokines and inflammatory mediators by metabolic disturbances is considered a core mechanism causing a chain of pathological processes in DR [5]. Representatives among them are IL (interleukin) -1b, IL-6, IL-8, IL-10, monocyte chemoattractant protein (MCP) -1, tumor necrosis factor-α (TNF-α), vascular endothelial growth factor (VEGF), and soluble VEGF receptor-1 (sVEGFR1 [6–12].

In response to a hypoxic state in chronically hyperglycemic DR eyes, hypoxic-driven angiogenic factors—particularly VEGF—lead to proliferation of NVs [13]. This NV tissue proliferates and subsequently develop a fibrous component, producing a firm adhesion between the retina and posterior hyaloid face [14, 15]. Localized traction from the posterior hyaloid face or contraction of the fibrous element of this fibrovascular complex leads to traction on the friable neovascular tissue and retina, progressing to vitreous hemorrhage [16]. IL-8, TNF-a, and MCP-1 as the major cytokines upregulated in eyes with PDR. This further stimulates fibrosis and vitreous contraction and, ultimately, results in traction retinal detachment [17].

Bleeding also leads to thrombotic activity. In addition to the known roles of coagulation and fibrinolytic cascades in thrombosis and hemostasis, these processes affect the inflammatory process [18]. The accumulation and activation of these and additional coagulation factors in the vitreous due to hemorrhage and chronic retinal injury in hemorrhagic PDR eyes may contribute to worsened retinal inflammation and capillary dysfunction, which leads to more severe retinal ischemia and edema [19].

Although hemorrhages frequently occur and are associated with advance stages of disease, the effects on DR progression are not fully understood. To the best of our knowledge, there is no existing study demonstrating hemorrhage in treatment naïve PDR and intraocular levels of cytokines and inflammatory mediators. Therefore, in this study, we attempted to elucidate the effect of hemorrhage in PDR eyes by investigating differences in cytokine profiles in PDR eyes with and without hemorrhage.

Materials and methods

This institutional retrospective cross-sectional comparative study was carried out in the Department of Ophthalmology at Bucheon St. Mary's Hospital, The Catholic University of Korea, Gyeonggi-do, Korea. This study was approved by the hospital's institutional review board and conducted according to the Declaration of Helsinki. Informed consent was obtained after the purpose and potential risks of the procedure were explained to each participant.

Patients

The study group consisted of patients with treatment-naïve PDR who agreed to participate in the study between August 2016 and September 2018. All patients included in the study were diagnosed with type 2 DM. PDR was manifested by posterior segment NV, which was defined by visible angiographic evidence as determined by modified Early Treatment Diabetic Retinopathy Study grade [20]. Enrolled eyes were divided into hemorrhagic and nonhemorrhagic groups based on presence of vitreous and/or preretinal hemorrhage. Exclusion criteria were as follows: (1) low-quality images due to significant cataract, corneal opacities, or poor cooperation; (2) eyes with diabetic macular edema [i.e., central macular thickness (CMT) of 350 μm or more and/or eyes with intra- or subretinal fluids] to eliminate interference in the cytokine system by macular edema (CMT was measured at earliest OCT available after resorption or removal of hemorrhage in cases of dense vitreous hemorrhage); (3) eyes with fibrous membrane and tractional retinal detachment; (4) eyes with iris NV and/or NV glaucoma; (5) receipt of any prior treatment for DR including anti-VEGF therapy, intraocular or periocular steroid, laser photocoagulation, or vitrectomy; and (6) presence of any significant retinal pathology other than PDR.

Each patient underwent a complete ophthalmologic examination, including measurement of best-corrected visual acuity (BCVA), slit-lamp examination, and dilated fundus examination. High-definition optical coherence tomography (HD-OCT) of the macula (Cirrus-HD 4000; Carl Zeiss Meditec, Jena, Germany) and mydriatic ultra-widefield color fundus photography and fluorescein angiography (Optos California P200DTx icg; Optos, Dunfermline, United Kingdom)

were performed. Demographic and clinical data including age, sex, coexistence of hypertension, duration of DM, and systolic and diastolic blood pressures were collected. Additionally, serum levels of random glucose, hemoglobin A1c (HbA1c), blood urea nitrogen (BUN), and creatinine (Cr) were recorded.

Image analysis

Central macular thickness as automatically obtained with A 6 × 6 mm area macular cube with the 512 × 128 protocol of the Cirrus HD-OCT system. The hemorrhage area was defined as any area blocked by vitreous or preretinal hemorrhage on ultra-widefield fluorescein angiography and was measured manually with Optos V2 software (Optos, Dunfermline, United Kingdom) (Fig. 1). The measured area was converted into unit of disk area, by dividing pixels of the area with pixels of the optic disk area.

Cytokine analysis

Undiluted samples of aqueous humor (0.1–0.2 mL) were aspirated by limbal paracentesis using a 30-gauge needle attached to a 1-cc syringe. The samples were placed into sterile tubes and immediately stored at − 80 °C in a deep freezer until analysis. Concentrations of IL-10, IL-1β, IL-6, IL-8, MCP-1, TNF-α, VEGF, and sVEGFR1 in aqueous humor samples were determined using the MILLIPLEX® MAP Human Cytokine/Chemokine Magnetic Bead Panel-Immunology Multiplex Assay (Millipore SAS, Molsheim, France).

Statistical analysis

Statistical analysis was performed using SPSS for Windows version 23.0.1 (IBM Corp., Armonk, NY, USA). For statistical analysis, the Snellen BCVA was converted to a logarithm of the minimal angle of resolution (logMAR). Values of continuous variables are presented as mean ± standard deviation. Independent *t* test was used to compare continuous variables between hemorrhagic and nonhemorrhagic eyes. The Mann–Whitney U test was used when a normal distribution could not be confirmed. Categorical variables were compared using the Chi-square test. Pearson's correlation analysis was used to determine the coefficients of correlation between clinical parameters and cytokine concentrations following confirmation of normal distribution. Spearman's correlation was used when normal distribution was not confirmed. A P value $<$ less than 0.05 was considered statistically significant.

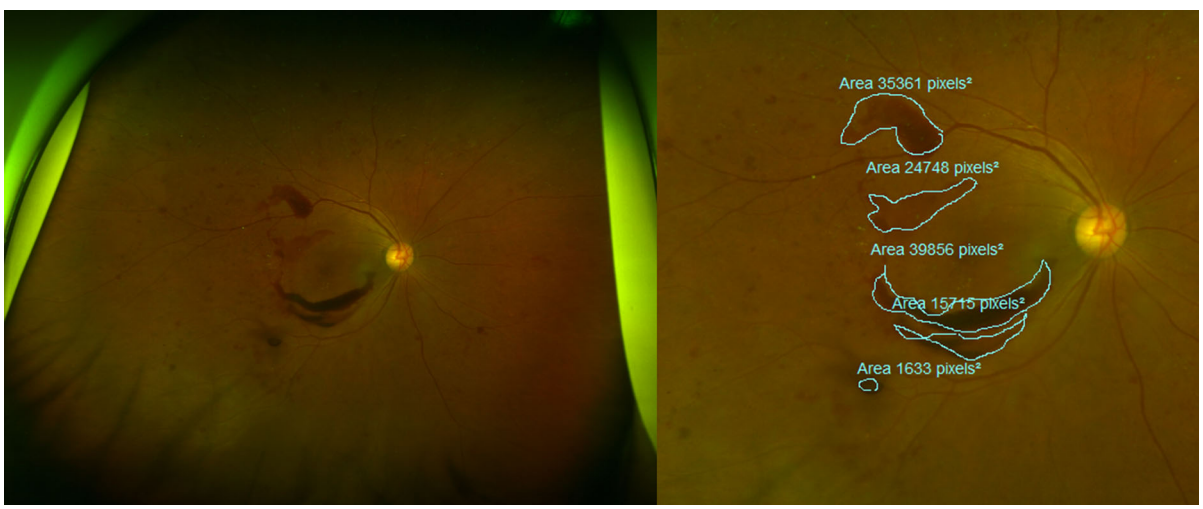

Fig. 1 Measurement of hemorrhage area. The hemorrhage area was defined as any area blocked by vitreous or preretinal hemorrhage on ultra-widefield fluorescein angiography and measured manually on Optos V2 software

Results

Demographic and clinical features

Sixty-six eyes of 42 type 2 DM patients with treatment-naïve PDR were enrolled. Eleven nonhemorrhagic eye were fellow eyes of hemorrhagic eyes. In total, 28 hemorrhagic and 38 nonhemorrhagic eyes were included in the study. All patients were Korean, and the mean age was 54.5 ± 10.4 (36–76) years. Fifty-three percent was male, and 76% had comorbid hypertension. The mean duration of DM was 15.10 ± 9.66 years. The mean BCVA was 0.28 ± 0.33 logMAR. Fourteen eyes (21%) were pseudophakic, and the lens status did not differ between hemorrhagic eyes (7 pseudophakia, 25%) and nonhemorrhagic (7 pseudophakia, 18%) eyes ($P = 0.364$).

The mean logMAR BCVA was better in the hemorrhagic group than in the nonhemorrhagic group (0.47 ± 0.38 vs. 0.14 ± 0.20; $P = 0.002$). Age, sex distribution, CMT, DM duration, random glucose, HbA1c, hypertension comorbidity, systolic and diastolic blood pressures, BUN, and Cr did not show significant differences between the groups ($P = 0.082$, 0.373, 0.881, 0.907, 0.237, 0.071, 0.437, 0.537, 0.978, 0.086, and 0.471). Baseline demographic and clinical features of study eyes are summarized in Table 1.

Cytokine concentrations and their association with hemorrhage

The mean aqueous humor concentrations of IL-8, MCP-1, TNF-α, and VEGF were higher in the hemorrhagic group than in the nonhemorrhagic group (12.93 ± 10.24 vs. 31.77 ± 51.22 pg/mL, 1382.88 ± 941.24 vs. 2210.23 ± 1461.95 pg/mL, 0.41 ± 0.18 vs. 0.64 ± 0.45 pg/mL, and 92.51 ± 119.13 vs. 213.13 ± 195.55 pg/mL; $P = 0.030$, 0.012. 0.006, and 0.003, respectively). No significant differences in IL-10, IL-1β, IL-6, and sVEGFR1 were observed between the groups ($P = 0.061$, 0.759, 0.486, and 0.303, respectively) (Table 2).

LogMAR BCVA exhibited positive correlation with concentrations of MCP-1, TNF-α, and VEGF ($r = 0.255$, 0.347, and 0.353; $P = 0.039$, 0.004, and 0.004, respectively) (Table 3). Further, logMAR BCVA demonstrated a positive correlation with hemorrhage area ($r = 0.494$; $P < 0.001$). Hemorrhage area was also positively correlated with concentrations of MCP-1, TNF-α and VEGF ($r = 0.253$, 0.474, and 0.648; $P = 0.040$, < 0.001, and < 0.001, respectively) (Table 3). When divided into subgroups, the correlation between hemorrhage area and concentration of TNF-α and VEGF remained significant in the

Table 1 Baseline demographic and clinical features of study eyes

Demographic features	Total ($n = 66$)	Nonhemorrhagic ($n = 38$)	Hemorrhagic ($n = 28$)	P value
Age (years)	54.5 ± 10.3	52.63 ± 10.81	57.04 ± 9.34	0.082
Gender (male/female)	35/31	19/19	16/12	0.373
Visual acuity (LogMAR)	0.28 ± 0.33	0.14 ± 0.20	0.47 ± 0.38	< 0.001*
CMT (um)	277.70 ± 44.10	276.97 ± 40.94	278.68 ± 48.82 ($n = 18$)	0.881
DM duration (years)	15.10 ± 9.66	14.97 ± 9.12	15.28 ± 10.53	0.907
Random glucose (mg/dl)	222.77 ± 111.48	238.91 ± 93.89	201.25 ± 130.36	0.237
HbA1c (%)	8.33 ± 2.14	8.78 ± 2.21	7.74 ± 1.94	0.071
HBP (n) (%)	50 (76)	28 (74)	22 (79)	0.437
BP systolic (mmHg)	138.88 ± 20.09	140.24 ± 22.04	137 ± 17.31	0.537
BP diastolic (mmHg)	78.33 ± 9.50	78.30 ± 9.18	78.38 ± 10.12	0.978
BUN (mg/dL)	17.87 ± 13.71	14.74 ± 5.32	22.04 ± 19.48	0.086
Cr (mg/dL)	1.00 ± 0.55	0.95 ± 0.58	1.06 ± 0.52	0.471

CMT, central macular thickness; *HbA1c,* glycated hemoglobin; *DM,* diabetes mellitus; *HBP,* hypertension; *BP,* blood pressure; *BUN,* blood urea nitrogen; *Cr,* creatinine

*Statistically significant P value

Table 2 Comparison of cytokine levels between hemorrhagic and nonhemorrhagic eyes

Cytokines	Total (n = 66)	Nonhemorrhagic (n = 38)	Hemorrhagic (n = 28)	P value
IL-10 (pg/mL)	1.55 ± 1.36	1.26 ± 1.14	1.93 ± 1.55	0.061
IL-1beta (pg/mL)	0.39 ± 0.08	0.39 ± 0.08	0.39 ± 0.07	0.759
IL-6 (pg/mL)	57.17 ± 180.07	44.38 ± 201.79	74.52 ± 147.36	0.486
IL-8 (pg/mL)	20.93 ± 35.18	12.93 ± 10.24	31.77 ± 51.22	0.030*
MCP-1 (pg/mL)	1733.88 ± 1249.75	1382.88 ± 941.24	2210.23 ± 1461.95	0.012*
TNF-α (pg/mL)	0.5 ± 0.34	0.41 ± 0.18	0.64 ± 0.45	0.006*
VEGF (pg/mL)	141.41 ± 166.57	92.51 ± 119.13	213.13 ± 195.55	0.003*
sVEGFR1 (pg/mL)	415 ± 238.56	388.06 ± 219.06	451.56 ± 262.37	0.303

IL, interleukin; *MCP-1,* monocyte chemoattractant protein- 1; *TNF-α,* tumor necrosis factor alpha; *VEGF,* vascular endothelial growth factor; *sVEGFR1,* soluble VEGF receptor 1

*Statistically significant P value

Table 3 Correlation between cytokine levels and area of hemorrhage/visual acuity

Variables	IL-10	IL-1beta	IL-6	IL-8	MCP-1	TNF-α	VEGF	sVEGFR1
BCVA								
Pearson Correlation	0.168	− 0.025	0.023	0.19	0.255	0.347	0.353	0.063
P value	0.178	0.843	0.855	0.127	0.039*	0.004*	0.004*	0.615
Area of hemorrhage								
Pearson Correlation	− 0.012	0.207	0.073	0.167	0.253	0.474	0.648	0.020
P value	0.926	0.095	0.563	0.181	0.040*	< 0.001*	< 0.001*	0.873

IL, interleukin; *MCP-1,* monocyte chemoattractant protein- 1; *TNF-α,* tumor necrosis factor alpha; *VEGF,* vascular endothelial growth factor; *sVEGFR1,* soluble VEGF receptor 1; *BCVA,* best-corrected visual acuity

*Statistically significant P value

hemorrhagic group (r = 0.426 and 0.718; P = 0.024 and < 0.001).

In comparison between hemorrhagic eyes and their fellow eyes, concentration of VEGF was higher in hemorrhagic eyes than fellow eyes (222.64 ± 67.13 vs. 205.45 ± 61.95 pg/mL, P = 0.005). Although the concentrations of IL-8, MCP-1, and TNF-α were higher in hemorrhagic eyes than fellow eyes, the values did not reach statistical significance (P = 0.217, 0.124, and 0.144, respectively, Table 4).

Discussion

Progression of PDR accompanies vitreous or preretinal hemorrhage, which is almost inevitable without proper treatment and systemic management in earlier stages. These hemorrhages may facilitate further progression of proliferative membrane formation and contraction [16]. Vitreous hemorrhage was the most common indication of NPDR and PDR progression in the Diabetic Retinopathy Clinical Research Network (DRCR) Protocol S post hoc study and at 2-year follow-up of Protocol T patients [21]. Nonetheless, there is not enough information on intraocular cytokine changes in hemorrhagic PDR eyes. In this study, we analyzed cytokine profiles in PDR eyes with and without hemorrhage, finding that aqueous humor levels of the inflammatory cytokines IL-8, MCP-1, and TNF-α and VEGF concentration were higher in hemorrhagic eyes compared to nonhemorrhagic eyes.

The aqueous concentration of VEGF was higher in hemorrhagic PDR eyes compared to nonhemorrhagic eyes. This result is consistent with findings of a previous study by Shinoda et al. [22], who reported that the level of aqueous humor was higher in PDR

Table 4 Comparison of cytokine levels between hemorrhagic eye and their fellow eyes

Cytokines	Hemorrhagic eyes (n = 11)	Fellow eyes (n = 11)	P value
IL-10 (pg/mL)	0.88 ± 0.26	1.33 ± 0.4	0.388
IL-1beta (pg/mL)	0.08 ± 0.02	0.1 ± 0.03	0.650
IL-6 (pg/mL)	102.52 ± 30.91	21.83 ± 6.58	0.110
IL-8 (pg/mL)	78.74 ± 23.74	10.43 ± 3.15	0.217
MCP-1 (pg/mL)	1871.9 ± 564.4	1121.04 ± 338.01	0.124
TNF-α (pg/mL)	0.59 ± 0.18	0.27 ± 0.08	0.144
VEGF (pg/mL)	222.64 ± 67.13	205.45 ± 61.95	0.005*
sVEGFR1 (pg/mL)	328.74 ± 99.12	277.34 ± 83.62	0.175

IL, interleukin; *MCP-1,* monocyte chemoattractant protein-1; *TNF-alpha,* tumor necrosis factor alpha; *VEGF,* vascular endothelial growth factor; *sVEGFR1,* soluble VEGF receptor 1

*Statistically significant P value

eyes with vitreous hemorrhage compared with among eyes without vitreous hemorrhage. In addition, a persistent increase in VEGF after vitrectomy was identified as a significant risk factor for postoperative early vitreous hemorrhage in patients with PDR [23]. This study adds a newer finding that amount of hemorrhage correlates with concentration of VEGF. Although the reason for elevated VEGF in hemorrhagic eyes is unclear, this serves as a rationale for vitrectomy in these eyes. Removal of vitreous hemorrhage to decrease VEGF may be beneficial in preventing progression and further complications of PDR.

Progression from moderately severe to severe NPDR to PDR with vitreous hemorrhage could be prevented by anti-VEGF treatment [24]. While some reports suggest that there is no conclusive evidence supporting the efficacy of anti-VEGF treatment in PDR [25], other studies have demonstrated benefits in absorption of hemorrhage [26]. In DRCR Protocol N, the ranibizumab group was more likely to complete panretinal photocoagulation without need for vitrectomy, and visual outcomes were more favorable in the ranibizumab group relative to in the saline group [27]. However, although there was no difference in rate of vitrectomy between hemorrhagic PDR eyes with or without anti-VEGF, the study was underpowered to detect a difference in vitrectomy rates due to the low overall rate of vitrectomies. Elevated VEGF level in hemorrhagic eyes can support use of anti-VEGF treatment for hemorrhage in PDR eyes.

Another important finding of this study was the elevation of the inflammatory cytokines IL-8, MCP-1, and TNF-α in hemorrhagic eyes. It had been suggested that the levels of IL-8 and MCP-1 are increased in the vitreous fluid of PDR eyes without hemorrhage and correlated with PDR activity [28]. The elevated levels of IL-8 and MCP-1 in hemorrhagic eyes may, therefore, be interpreted as more severe stages of PDR in these eyes. Alternatively, this may imply increase in inflammatory activity in hemorrhagic eyes, regarding that there was no difference between demographic and clinical features between groups. In addition, correlation of these cytokines with visual acuity and hemorrhage area can indicate that inflammation is more significant with larger amounts of hemorrhage. Although it is not possible to determine whether this is a cause or result of hemorrhage, this finding has its significance in that it indicates that the ongoing inflammatory process involving these cytokines is more active in PDR eyes with hemorrhage.

It is known that blood stimulates an inflammatory reaction in the extravascular space as part of the reparative process [29]. Acute hemolysis increases blood cytokine levels in humans [30]. Elsewhere, hemoglobin formed by hemolysis was shown to increase IL-8 release by polymorphonuclear phagocytes and to augment TNF-α release by macrophages [31]. TNF-α is also known to be elevated in subarachnoid hemorrhage of the brain [32]. This can be applied in the case of hemorrhage in the vitreous cavity. Inflammation is required for removal of hemorrhage from the vitreous cavity by facilitating

erythrophagocytosis with macrophage action [29]. The inflammatory responses of allergic uveitis and infection are reported to accelerate removal of intravitreal blood [33]. Additionally, the thrombotic activity that occurs following hemorrhage may induce the inflammatory process. Plasma kallikrein, thrombin, and urokinase are increased in DR and exert proinflammatory effects that contribute to retinal vascular dysfunction [19].

While inflammation seems mandatory in resorption of hemorrhage, the presence of high levels of IL-8, MCP-1, and TNF-α may promote to progression of PDR. High levels of IL-8 and TNF-α in the aqueous humor may be associated with progression of NV [34]. The level of IL-8 at the time of vitrectomy was associated with occurrence of recurrent hemorrhage after surgery [35]. Together with the high level of VEGF, which is known to be the cause of NV after vitrectomy, this may increase the possibility of recurrent hemorrhage in hemorrhagic PDR eyes [36]. Moreover, elevated levels of MCP-1 and IL-8 can be the cause of postoperative fibrous proliferation and may eventually promote tractional retinal detachment in PDR eyes [36]. The MCP-1 level was markedly elevated at the second vitrectomy, implying an association between prolonged inflammation after vitrectomy and complications, especially tractional retinal detachment [37]. MCP-1 also is known to be present in the vast majority of eyes affected by PVR. Based on these, we carefully infer that prevention of recurrent hemorrhage using intraoperative anti-VEGF during vitrectomy for PDR might reduce further unnecessary inflammatory process caused during the resorption of hemorrhage [38].

This study has inherent limitations owing to its cross-sectional design. It is impossible to detect whether the result observed in this study is the cause or effect of hemorrhage, as mentioned above. A prospective study including follow-up data of before and after hemorrhage in PDR eyes is required to solve this problem. And, the hemorrhagic area could be under-estimated in cases of preretinal hemorrhage, since preretinal hemorrhage can spread to larger vitreous hemorrhage in the presence of posterior vitreous detachment. Further, although we excluded eyes with macular edema to focus on the effect of hemorrhage in cytokines, there were a few cases where the hemorrhage was too large to successfully detect macular edema. Follow-up of these two eyes did not reveal clinically significant macular edema nor hard exudate. Most importantly, the sample size of the current study was relatively small, especially for comparison between hemorrhagic and fellow eyes; therefore, research with larger sample sizes is required to validate the results of this study.

Conclusions

In summary, the results of this study support that the presence of hemorrhage in PDR was associated not only with aqueous humor concentration of VEGF but also with the levels of inflammatory cytokines such as IL-8, MCP-1, and TNF-α. These cytokines also showed positive correlations with the amount of vitreous hemorrhage, suggesting roles of VEGF and inflammation in hemorrhagic eyes. Elevation of VEGF in these eyes may suggest a role of anti-VEGF treatment in hemorrhagic PDR. Suppression of the inflammatory process should be considered cautiously as it helps in promoting resorption of hemorrhage, although the same process may also lead to fibrous proliferation and tractional retinal detachment.

Author contributions Contributions were as follows: HR involved in preparation of data and data analysis; AL involved in conceptualization and data analysis; JHL and IKK participated in collection of data and data analysis; JB participated in conception and design of the study, writing manuscript text, preparing figures, collection and assembly of data, data analysis and interpretation, and supervision. All authors reviewed the manuscript.

Funding This work was supported by the Institute of Clinical Medicine Research of Bucheon St. Mary's Hospital Research Fund, 2019 and a grant from the Korea Health Technology R&D Project through the Korea Health Industry Development Institute, funded by the Ministry of Health and Welfare, Republic of Korea (Grant no: HI17C2012030018).

Data availability The datasets generated and/or analyzed during the current study are available from the corresponding author upon reasonable request.

Compliance with ethical standards

Conflicts of interest The authors have no competing interests to declare.

Consent to participate Informed consent was obtained after the purpose and potential risks of the procedure were explained to each participant.

Consent for publication The authors agree to transfer the publication rights to the journal.

Ethics approval This study was approved by the institutional review board of Bucheon St. Mary's hospital and conducted according to the Declaration of Helsinki.

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