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Role of Soluble Vascular Endothelial Growth Factor Receptor-1 and -2 in Regulating Branch Retinal Vein Occlusion with Macular Edema

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PX and LZ collected the samples. MD and HZ analysed the data. FC conducted the experiments and analysed the results. All authors discussed the results and wrote the manuscript.

Key words
Vascular endothelial growth factor receptor, Macular edema, Cytokines, Pro-inflammatory factors

ABSTRACT

The objective of this study was to investigate the mechanism of soluble vascular endothelial growth factor receptor-1 (sVEGFR-1) and sVEGFR-2 in regulating branch retinal vein occlusion with macular edema. A total of 45 patients with branch retinal vein occlusion and macular edema who were treated at Tangshan Eye Hospital from November 2018 to June 2020 were enrolled in the study, including 19 cases of localized macular edema, 12 cases of diffuse macular edema, and 14 cases of cystoid macular edema. Forty-five healthy people in physical examination were recruited as control. We found that the diseased group had higher proportion of hypertension (P<0.05) compared with the control group, and there was no difference in other general information (gender, age, BMI) (P>0.05). Compared with the control group, the diseased group had higher expression of sVEGFR-1 and sVEGFR-2 mRNA (P<0.05), and higher content of VEGF, PlGF, sICAM-1, MCP-1 and PDGF than the control group (P<0.05). The diseased group had higher protein expression of IL-6, IL-8, IL-12 and IL-13 than the control group (P<0.05). In detection of protein expression levels of sVEGFR-1 and sVEGFR-2 in patients with different severity levels, diffuse macular edema group had higher protein expression levels of sVEGFR-1 and sVEGFR-2 than the localized macular edema group (P<0.05), and cystoid macular edema group had higher protein expression levels of sVEGFR-1 and sVEGFR-2 than diffuse macular edema group (P<0.05). In the case of more severe macular edema, the sVEGFR-1 and sVEGFR-2 protein expression levels are higher. It is concluded that the aqueous humor levels of factors associated with branch retinal vein occlusion with macular edema, such as growth factors, sVEGFR-1, sVEGFR-2 and inflammatory factors, are increased, indicating that there is a very important relationship between cytokine networks, which can help us better understand the disease mechanism and develop new treatment methods.

INTRODUCTION

Branch retinal vein occlusion is a common retinal disease which mainly occurs in elderly patients (Kaldırım and Yazgan, 2018). Without therapeutic intervention, two-thirds of patients will further develop macular edema which causes permanent visual impairment (Iijima, 2018). Branch retinal vein occlusion originates from vascular occlusion associated with atherosclerosis, but the disease process involves a combination of mechanical, ischemic and inflammatory changes. It has been reported that retinal ischemia, vascular remodeling and atherosclerosis are all related to inflammation (Simsek *et al*., 2018). These processes are all guided by inflammatory cytokines, chemokines and growth factors that control the behavior of lymphocytes, macrophages and bone marrow-derived endothelial progenitor cells (Khayat *et al*., 2018). The pathogenesis of macular edema in branch retinal vein occlusion is mediated by a variety of angiogenesis and inflammatory cytokines, especially vascular endothelial growth factor (VEGF) (Sengupta and Pan, 2017). Therefore, the pathogenesis and progression of branch retinal vein occlusion in patients with macular edema may be mediated by several intraocular angiogenesis and inflammatory mediators (Campagnoli *et al*., 2017). It has been reported that intravitreal injection of bevacizumab (a monoclonal antibody against VEGF) and ranibizumab (which binds and neutralizes Fab fragments of all isoforms in VEGF-A) can alleviate macular edema in these patients

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(Kwon *et al*., 2018). sVEGFR-2 may be involved in the pathogenesis of macular edema associated with branch retinal vein occlusion (Kwon *et al*., 2018). Recently, it was found that intravitreal injection of aflibercept (VEGF Trap-Eye, a fusion protein which binds the key domains of human VEGFR-1 and VEGFR-2 with the constant region (Fc) of human immunoglobulin G, and binds several VEGF-A subtypes) can improve macular edema in patients with central retinal vein occlusion (Cehofski *et al*., 2018). Therefore, various factors including VEGFR-1 and VEGFR-2 may interact to produce macular edema associated with branch retinal vein occlusion, but this process still remains unclear (Georgalas *et al*., 2019). We measured the aqueous humor levels of 11 factors (including VEGFR, growth factors and inflammatory factors) in patients with branch retinal vein occlusion macular edema and cataract patients as the control, and then analyzed the severity of each factor and macular edema.

MATERIALS AND METHODS

General information

A total of 45 patients with branch retinal vein occlusion and macular edema who were treated at Tangshan Eye Hospital from November 2018 to June 2020 were enrolled in the study, including 19 cases of localized macular edema, 12 cases of diffuse macular edema, and 14 cases of cystoid macular edema. Forty-five healthy people in physical examination were recruited as the research control. The subjects were divided into control group and diseased group. The diseased group was divided into localized macular edema group, diffuse macular edema group, and cystoid macular edema group according to the severity of macular edema.

Inclusion criteria: gender and age are unlimited; those diagnosed with branch retinal vein occlusion with macular edema.

Those with history of retinal diseases other than glaucoma, uveitis, diabetes, erythema iris, eye infection, laser photocoagulation and intraocular surgery (including cataract surgery); patients with incomplete data.

The study was conducted in accordance with the Declaration of Helsinki and was approved by the ethics committee of the hospital. All patients signed an informed consent form before inclusion.

Patient eye examination

The graders independently assessed ischemic retinal vascular occlusion by examining fluorescein angiography. As mentioned earlier, the ischemic area of the retina was measured using the public domain Scion Image program. On the digital fundus photograph, the intervertebral disc area was outlined with cursor, and then measured. The same operation was performed on the non-perfusion area. Severity of retinal ischemia was assessed by dividing the unperfused area by the area of the intervertebral disc. Within 1 week before the intravitreal injection of bevacizumab, optical coherence tomography was performed in each subject using a spectral domain optical coherence tomography device (Fig. 1). Severity of macular edema was classified according to the thickness of central macula, neurosensory retinal thickness, and subfoveal serous retinal thickness. Measurements of these parameters were as follows: The central macular thickness was calculated as the distance from the inner limit membrane of the retinal pigment epithelium to the basement membrane (including all compartments between them). The neurosensory retinal thickness is thickness of faveolus neurosensory retina and the subfoveal serous retinal thickness is the serous membrane thickness of the fovea. Two retinal experts measured the calipers of the Caterpillar machine, who had no idea of the subject's BCVA status and cytokine levels.

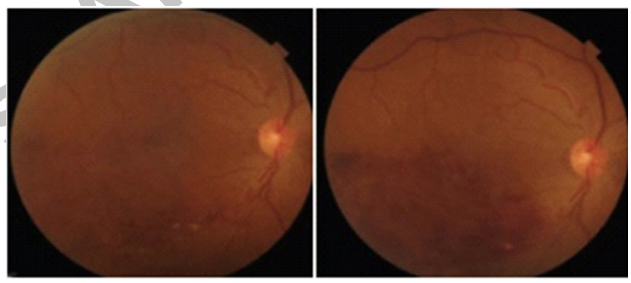

Fig. 1. Optical coherence tomography of macular edema.

Sample collection

All patients with branch retinal vein occlusion received intravitreal injection of 1.25 mg bevacizumab. A sample of aqueous humor was collected during the intravitreal injection. A 30-gauge needle connected to an insulin syringe was used to collect 0.1 mL of aqueous humor through corneal limbus puncture of anterior chamber. Then intravitreal injection of bevacizumab was given through the intraperitoneal cavity 3.5 mm from the marginal edge. Antibiotic ointment was given 7 days after surgery. Immediately after collection, the aqueous humor sample was transferred to a sterile plastic tube and stored at -80°C until analysis. Control water samples were collected from 9 patients undergoing routine cataract surgery by corneal limbal puncture, and then frozen and stored at -80°C.

Real-time quantitative RT-PCR

Based on ABIPRISM®7700 sequence detection

system, real-time quantitative RT-PCR was used to evaluate mRNA levels of VEGF-R1 and VEGF-R2. The real-time RT-PCR conditions have been described above. In short, the reaction was performed in triplicate, each containing 1 µl cDNA and 2.5 pmoles gene-specific primers, and the final volume was 25 µl. The thermal characteristics were as follows: a 5-min initial denaturation was performed to activate the DNA polymerase, for 35 seconds at 95°C, 45 seconds at 60°C and 45 seconds at 72°C, respectively, each with 40 cycles. GAPDH was selected as reference gene. Each sample was subjected to PCR in triplicate.

Estimation of cytokines and growth factors

The samples were analyzed using suspension array technology. Kit was used for detection of sVEGFR-1, sVEGFR-2, VEGF, placental growth factor (PlGF), soluble intercellular adhesion molecule (sICAM)-1, monocyte chemotactic protein 1 (MCP-1), platelet derived growth factor (PDGF). Samples of undiluted aqueous humor (25µL) were incubated overnight (16-18 h) to detect PlGF and *sIC*AM1, or for 2 h to measure other factors. The kit was used according to the manufacturer's instructions. A standard curve (in duplicate) for each cytokine was generated by using the reference set of cytokine concentration provided in each kit. All incubation steps were performed at room temperature and in the dark. Samples were read on the suspension array system. To avoid inaccuracies between two runs, we measured cytokines in all patient samples in one run. Control samples were used in all runs, and the levels of these factors in aqueous humor samples were within the detection range of the assay.

Western blot analysis

The aqueous humor sample was dissolve and the total protein was extractd using the whole cell protein extraction kit (Beijing, China) according to the manufacturer's protocol. The membrane was blocked with 2% BSA in TBST for 2 h at room temperature. Then the membrane was incubated with the primary antibody overnight at 4°C. The main antibodies used in our study included rabbit anti-IL-6 (1:1000), IL-8 (1:1000), IL-12 (1:1000), IL-13 (1:1000), and GAPDH (1:4000). Then the PVDF membrane was washed 3 times with TBST and developed with ECL Plus reagent. The optical density of each protein was analyzed using Image J software.

Statistical analysis

SAS System 9.3 software was used for analysis. Student's t test was used to compare normally distributed unpaired continuous variables between the two groups, and Mann-Whitney U test was used for comparison. Discrete variables were compared using χ^2 test or Fisher's exact test. The difference between median water levels was relatively high in Wilcoxon single rank test. According to calculation using Spearman's rank correlation coefficient, the two-tail *P* value was less than 0.05, indicating statistical significance.

RESULTS

Table I shows general information of patients involved in this study. There were 27 male and 18 female patients, with an average age of 67.58±8.43, smoking 12 (26.66%), and hypertension 28 (62.22%). Compared with the control group, the diseased group had higher proportion of hypertension (P<0.05).

Table II shows the expression of sVEGFR-1 and sVEGFR-2 mRNA in serum by PCR real time analysis, the level of cytokines by suspensions array technology, expersion of proinflammatory factors by western blot analysis and protein expression levels of sVEGFR-1 and sVEGFR-2 of control and diseased patients. The diseased group had higher expression of sVEGFR-1 and sVEGFR-2 mRNA than the control group (*P*<0.05) (Fig. 2) higher content of VEGF, PlGF, sICAM-1, MCP-1, PDGF in the diseased group than the control group (*P*<0.05) and higher protein expression of IL-6, IL-8, IL-12, and IL-13 than the control group (*P*<0.05). Table III shows the protein expression levels of sVEGFR-1 and sVEGFR-2 in patients with different severity levels, the diffuse macular edema group has higher protein expression levels of sVEGFR-1 and sVEGFR-2 than the localized macular edema group (*P*<0.05), and cystoid macular edema group has higher protein expression levels of sVEGFR-1 and sVEGFR-2 than the diffuse macular edema group (*P*<0.05). With more severe macular edema, sVEGFR-1 and sVEGFR-2 protein expression levels are higher as show in Figure 3.

Table IV shows linear correlation analysis patients' sVEGFR-1 and sVEGFR-2 protein expression of serum growth factor, IL-6, IL-8, IL-12, IL-13 levels of pro-inflammatory factors and VEGF, PlGF, sICAM-1, MCP-1, PDGF levels of other cytokines which are significantly correlated with branch retinal vein occlusion with macular edema (*P*<0.05).

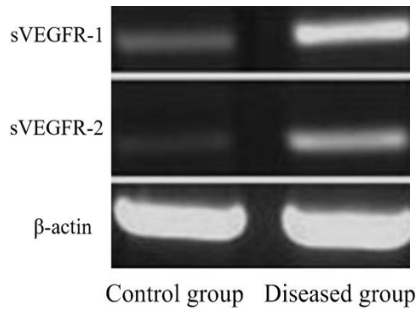

Fig. 2. Real-time PCR analysis.

Table I. General data statistics.

Variable	Gender (male: female)	Age	BMI	Hypertension (n, %)	Smoking (n, %)
Control group	21:24	66.34±9.28	23.16±1.74	5 (11.11%)	8 (17.78%)
Diseased group	27:18	67.58±8.43	22.73±2.06	28 (62.22%)	12 (26.66%)
t value	5.327	4.162	6.127	4.115	5.033
P value	0.268	0.635	0.219	0.025	0.141

Table II. Expression of sVEGFR-1 and sVEGFR-2 in serum by RT-PCR, levels of cytokines by suspension assay, expression of pro inflammatory factors by western blot analysis, and protein expression of sVEG FR-1 and sVEGFR-2 control and diseased patients.

Group	Control group	Diseased group	t value	*P* value
mRNA level in serum				
sVEGFR-1	1.02±0.13	1.94±0.35	5.037	0.026
sVEGFR-2	0.86±0.10	2.03±0.54	6.382	0.001
Cytokines in aqueous humor pg/mL				
VEGF	19.36±6.57	81.69±17.42	4.328	0.012
PlGF	0.23±0.05	1.74±0.28	5.014	0.003
sICAM-1	0.05±0.01	0.31±0.012	6.335	0.035
MCP-1	765±86.77	1835±263.88	4.167	0.028
PDGF	14.27±2.43	32.91±5.70	5.237	0.019
Protein expression of pro-inflammatory factors (±s)				
IL-6	1.14±0.23	2.21±0.36	4.167	0.036
IL-8	1.02±0.12	1.86±0.23	5.328	0.004
IL-12	0.93±0.11	1.74±0.20	6.091	0.028
IL-13	1.05±0.22	1.96±0.35	5.135	0.018

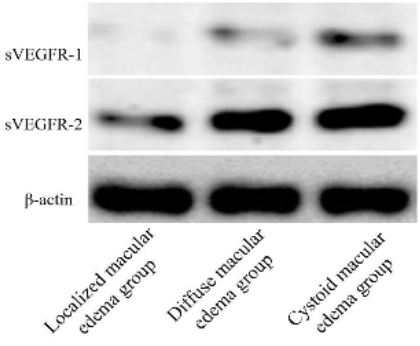

Fig. 3. Western blot analysis of sVEGFR-1 and sVEGFR-2 protein.

Table III. Detection of sVEGFR-1 and sVEGFR-2 protein levels in different groups pg/mL.

Variable	Localized macular edema group	Diffuse macular edema group	Cystoid macular edema group	F value	*P* value
sVEGFR-1	0.135±0.01	0.168±0.02	0.193±0.03	9.267	0.033
sVEGFR-2	0.164±0.02	0.185±0.03	0.197±0.04	11.627	0.018

Table IV. Detection of sVEGFR-1 and sVEGFR-2 protein levels in different groups pg/mL(±s).

Factor	Branch retinal vein occlusion with macular edema	
	r	p
sVEGFR-1	0.264	0.015
sVEGFR-2	0.136	0.026
IL-6	0.375	0.003
IL-8	0.241	0.002
IL-12	0.326	0.014
PlGF	0.119	0.005
PDGF	0.268	0.034
sICAM-1	0.357	0.026
VEGF	0.294	0.004
MCP-1	0.315	0.018

DISCUSSION

In this study, we proved that the diseased group had higher aqueous humor levels in sVEGFR-1 and sVEGFR-2 than the control group, and there was a significant correlation between the levels of sVEGFR-1 and sVEGFR. Soluble VEGFR-1 and -2 are produced by alternative mRNA splicing, which allows the same gene to encode the transmembrane form of VEGFR-1 and VEGFR-219-21 or the soluble form released from the cell surface (Chung and Li, 2018). VEGFR1 is not only expressed in vascular endothelial cells, but also expressed

by monocytes/macrophages at the mRNA and protein levels, which plays a role in the recruitment of these cells together with VEGFR-1 signal, thereby generating VEGF in blood vessels and inflammation sites (Wu *et al*., 2017). On the other hand, VEGFR-2 is only expressed by endothelial cells, and VEGF signal transduction mediated through the membrane-bound isoform of VEGFR-2 is essential for normal endothelial cell function, which affects vascular permeability and angiogenesis (Wu *et al*., 2017).

Generally, soluble receptors bind with ligands to inactivate their ligands, which is because soluble receptors do not have the intracellular domain required to initiate signal transduction (Khurana *et al*., 2018). That is to say, sVEGFR-1 acts as a decoy for VEGF (endogenous antagonist) and as a negative regulator of angiogenesis and reperfusion after ischemia (He *et al*., 2018). Both in vitro and in vivo studies have shown that elevated levels of sVEGFR-1 can impair vasodilation response, while sVEGFR-2 has anti-angiogenic activity (Nikkhah *et al*., 2018). This study reveals that the severity of macular edema (CMT or SRT) is positively correlated with the levels of sVEGFR-1 and sVEGFR-2 in aqueous humor, which indicates that whether sVEGFR-1 and sVEGFR-2 bind with VEGF to neutralize its effect on vascular endothelium has relation to vascular permeability and occurrence of macular edema. It is reported that macular edema can be deteriorated by increasing VEGF bound to receptors expressed on vascular endothelial cells, monocytes and macrophages (Kaldırım and Yazgan, 2018). Interestingly, sVEGFR-1 has also been reported to promote inflammation (Dong *et al*., 2018). In addition, we previously suggested inducing multiple inflammatory factors via NF-κβ based on our finding that the vitreous humor level of sVEGFR-2 was significantly correlated with the levels of several inflammatory factors including sICAM-1, MCP-1 and IL-6. This study shows that the aqueous humor level of sVEGFR-2 is significantly correlated with the levels of various inflammatory factors (sICAM-1, MCP-1, IL-6 and IL-8), as was previously found in the vitreous level. Clinical and experimental evidence shows that both sVEGFR-1 and sVEGFR-2 can affect vascular permeability in the inflammatory response. However, further studies are needed to confirm the role of sVEGFR-1 and sVEGFR-2 in macular edema associated with branch retinal vein occlusion. We also found that the levels of PlGF and PDGF-AA were higher in the aqueous humor samples of the branch retinal vein occlusion group than in the control group. This result is consistent with previous reports that PDGF-AA and PlGF are overexpressed in the eye fluid of patients with retinal diseases (such as RVO, diabetic retinopathy and age-related macular degeneration) (Hosogi *et al*., 2018). In addition to VEGF, PlGF is also a ligand of VEGFR-1, so these two molecules can both play a role in signal transduction under pathological conditions (Lucatto *et al*., 2017). After it binds to VEGFR-1 through a calcineurin-dependent pathway, it also increases the production of IL-8 and MCP-1 through cultured monocytes, suggesting that it has a direct impact on the inflammatory response. Our findings support this view, that is, the aqueous humor levels of PlGF are correlated with the aqueous humor levels of sVEGFR-1, MCP-1 and IL-8. In addition, it is reported that PlGF induces monocytes to secrete VEGF. Our findings support this point, that is, the aqueous humor level of PlGF is significantly correlated with the aqueous humor level of VEGF (Gutkowski *et al*., 2011).

We have previously reported that compared with the control group, the levels of three inflammatory factors (sICAM-1, MCP-1 and IL-6) increased in the vitreous humor samples of patients with branch retinal vein occlusion, and these inflammatory factors are significantly correlated with each other (Mou *et al*., 2020). These inflammatory factors play an important role in the occurrence of macular edema associated with branch retinal vein occlusion. Similarly, in this study, compared with the control group, the inflammatory factors (IL-6, IL-8, sICAM-1 and MCP-1) have higher levels in the aqueous humor samples of the branch retinal vein occlusion group, and most inflammatory factors are significantly correlated with each other. It has been reported that the concentrations of IL-6 and IL-8 were significantly higher in aqueous humor of patients with retinal vein occlusion than in the control group. Their report is consistent with the results of this study. They also reported that IL-6 levels were significantly correlated with CMT. It is reported that IL-8 levels are positively correlated with the severity of macular edema and retinal ischemia in patients with branch retinal vein occlusion and macular edema. Interleukin 8 is a potent chemokine and an activator of neutrophils and T cells. The production of interleukin 8 is induced by exposure of vascular endothelial cells to a hypoxic state, and then the cytokine is exposed to angiogenesis and tumor metastasis. There are also reports that IL-8 can increase vascular permeability. These reports plus our findings suggest that IL-8 may be the main inflammatory factor for macular edema associated with branch retinal vein occlusion.

CONCLUSION

In conclusion, the aqueous humor levels of various factors (growth factors, sVEGFR-1, sVEGFR-2 and inflammatory factors) were higher in eyes with branch retinal vein occlusion than in the control group. The levels

of sVEGFR-1 and -2 are correlated with the levels of SRT, growth factors (PlGF and PDGF-AA) and various inflammatory factors. Significant correlation is also shown with the levels of growth factors (VEGF, PlGF and PDGF-AA). These findings indicate the importance of studying the relationship between cytokine networks as it may help us better understand the mechanism of macular edema in patients with branch retinal vein occlusion and then develop new treatment methods.

Statement of conflict of interest

The authors have declared no conflict of interest.

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