Profile of vascular and inflammatory aqueous humor cytokines in primary open angle glaucoma patients

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Ruyi Zha  
Eye, Ear, Nose and Throat Hospital, Shanghai Medical College, Fudan University  
ORCiD: 0000-0002-7906-6357

Huan Xu  
Eye, Ear, Nose and Throat Hospital, Shanghai Medical College, Fudan University

Xinghuai Sun  
Eye, Ear, Nose and Throat Hospital, Shanghai Medical College, Fudan University

Xiangmei Kong  
Eye, Ear, Nose and Throat Hospital, Shanghai Medical College, Fudan University  
kongxm95@163.com Corresponding Author  
ORCiD: 0000-0002-8142-6040

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Abstract

**Purpose:** This study was aimed to analyze the profile of vascular and inflammatory cytokines in aqueous humor of primary open angle glaucoma (POAG) patients.

**Methods:** Aqueous humor samples were acquired from 6 POAG patients and 7 age-related cataract patients that constituted the control group. A human cytokine antibody array was used to detect 37 proteins related to inflammation and vascular regulation. Characteristics including age, gender, and intraocular pressure (IOP) of POAG and age-related cataract patients were compared. Correlation analyses between aqueous humor cytokines and characteristics were performed.

**Results:** The cytokine antibody array results showed that the signal intensities of soluble vascular endothelial growth factor receptor 1 (sVEGFR-1), von Willebrand factor (vWF), tumor necrosis factor (TNF)-α, and angiopoietin-2 in POAG patients were significantly higher in aqueous samples compared to controls (p < 0.05, Student’s test or Mann-Whitney U-test). Among cytokines of all aqueous samples, preoperative IOP was positively associated with expression quantity of sVEGFR-1 (r = 0.647, p = 0.02) and VEGF-A (r = 0.602, p = 0.04) and negatively associated with plasminogen activator inhibitor 1 expression (r = -0.593, p = 0.04). Values for sVEGFR-1 were positively correlated with vWF (r = 0.646, p = 0.02) and TNF-α (r = 0.824, p < 0.001) with statistical significance.

**Conclusion:** Pathogenesis of primary open angle glaucoma may be associated with both vascular dysfunction and inflammatory responses. The vascular factors may include endothelial dysfunction and damaged vascular permeability as indicated by abnormal expression of vWF, sVEGFR-1 and angiopoietin-2.

**Keywords:** POAG, aqueous humor, cytokines, sVEGFR-1, vWF, angiopoietin-2

Introduction
Glaucoma, a neurodegenerative disease, is the leading cause of irreversible blindness worldwide [1]. The pathogenesis of primary open angle glaucoma (POAG), one of the most common types of glaucoma, has been in controversy including various hypotheses, which mainly included mechanical pressure theory [2], vascular hemodynamic theory [3] and inflammatory theory. Elevated intraocular pressure (IOP) is considered one of the most crucial factors in pathogenesis of POAG. In POAG, raised IOP is usually due to increased outflow resistance in trabecular meshwork. Researchers observed an elevated amount of inflammatory cells in trabecular meshwork of glaucoma patients, which suggests glaucoma is associated with inflammatory responses. Besides, in some glaucomatous patients with open angle and normal IOP or some POAG patients with well-controlled IOP, the disease is still progressing, indicating that there are other risk factors. Based on it, vascular factors have been postulated to play a role in glaucoma progression, including vasospastic or organic alterations. To date, the exact pathogenesis of POAG has not been expounded.

The outflow of aqueous humor exerts a crucial influence on IOP maintenance and the occlusion of aqueous drainage could lead to elevated IOP levels. There are abundant cytokines in aqueous humor, altered in and also reflecting pathological conditions. Alterations of cytokine levels have been observed in and associated with certain ocular diseases, including diabetic retinopathy [4], central retinal vein occlusion [5], age-related cataract [6] and macular edema [7]. Several studies have detected increased levels of some cytokines in glaucoma aqueous humor, including inflammatory and vascular cytokines [8-10]. However, the profile of aqueous humor cytokines has not been fully studied and the role of aqueous humor cytokines present in POAG patients is still unclear. Furthermore, due to the limitations of conventional enzyme-linked immunosorbent assay (ELISA) methods, correlations among cytokines are difficult to evaluate in a single
experiment. Cytokine antibody arrays make it possible to investigate multiple aqueous cytokines in one sample simultaneously [6,11,12]; consequently, associations between different cytokines can be studied. Therefore, it is necessary to profile the vascular and inflammatory cytokines in aqueous humor in order to investigate the possible pathophysiological mechanism in POAG associated with vascular and inflammatory changes.

The aim of this study is to investigate the profile of vascular-associated and inflammatory cytokines in the aqueous humor of POAG patients and elucidate their roles in POAG pathogenesis. Moreover, correlations among different cytokines and between cytokines and clinical characteristics are analyzed.

Methods
This case-controlled study was approved by the medical ethics committee of the Eye and Ear Nose and Throat Hospital of Fudan University. All enrolled patients submitted informed written consent after receiving a careful explanation of the study. This study was performed in accordance with the Declaration of Helsinki.

Subjects
Case samples were collected from 6 eyes of 6 POAG patients. Inclusion criteria for POAG group including 1) age ≥20 years old. 2) In accordance with the diagnosis of POAG. Diagnosis of POAG is based on an open anterior chamber angle with a gonioscopy examination, glaucomatous optic nerve damage (no less than two of the following presences: cup-disk ratio≥0.6, regional rim loss, deficits of nerve fiber layer or disc haemorrhage) and typical visual field defects. Patients with any systemic diseases such as autoimmune disorders and diabetes mellitus, ocular diseases such as high myopia,
pseudoexfoliation syndrome, and uveitis, as well as any glaucoma types other than POAG were excluded. Seven eyes of 7 age-related cataract patients were included as controls; samples were collected before surgical interventions for cataracts. Exclusion criteria for control patients included the following: a diagnosis of glaucoma, pseudoexfoliation syndrome, uveitis, diabetes, a history of previous filtration surgery, ocular inflammation, ocular trauma, and ocular ischemia. All patients completed appropriate examinations including best-corrected visual acuity (BCVA, E charts at a distance of 5 m), IOP tests (Goldmann tonometry, GAT, Haag Streit AG, Bern, Switzerland), slit-lamp biomicroscopy (Type YZ5E, 66 Vision Tech. Co., China), gonioscopy (Goldmann one-mirror lens, Haag Streit, Bern, Switzerland) and Lenstar test 5 to 21 days before surgery. POAG patients accepted a Humphrey 30-2 computerized visual field examination (Carl Zeiss Meditech, Inc., Dublin). All surgeries and experiments were conducted from May to November 2016.

Sample collection

Immediately before surgery, a medical professional applied topical anesthetics (0.4% oxybuprocaine, Santen, Osaka, Japan) to all POAG and cataract patients’ eyelids and surrounding skin. Next, aqueous humor samples were collected (100-150 μl) through corneal paracentesis by inserting a 26 gauge needle under local anesthesia. Samples were immediately stored at -80°C.

Cytokine antibody array

Our study analyzed a selection of 37 cytokines simultaneously by using an antibody-based cytokine array (Raybiotech, Norcross, GA, USA) to investigate the alteration of cytokines
in aqueous humor samples including vascular endothelial growth factor (VEGF)-A, VEGF-C, VEGF-D, soluble VEGF receptor 1 (sVEGFR-1), sVEGFR-R3, von Willebrand factor (vWF), endothelin-1, placental growth factor (PIGF), platelet derived growth factor (PDGF)-AB, PDGF-BB, stromal cell-derived factor (SDF)-1α, SDF-1β, angiopoietin-1, angiopoietin-2, angiostatin, angiopoietin-like 4 (ANGPTL4), Tie-1, Tie-2, erythropoietin (EPO), monocyte chemoattractant protein (CCL2/MCP)-1, C-C chemokine ligand 3/macroage inflammatory protein (CCL3/MIP)-1α, CCL4/MIP-1β, C-C chemokine ligand 5/regulated upon activation, normal t cell expressed, and secreted (CCL5/RANTES), endoglin, fas, fas-ligand (fas-L), intercellular adhesion molecule (ICAM)-1, interleukin (IL)-1b, IL-6, IL-8, IL-12 (p70), matrix metalloproteinase (MMP)-1, MMP-9, plasminogen activator inhibitor (PAI)-1, tissue inhibitor of metalloproteinase (TIMP)-1, TIMP-2, and tumor necrosis factor (TNF)-α. The antibody array experiment, based on the sandwich immunoassay principle, was conducted according to manufacturer’s protocol. After samples (80 μl) were applied to each block, antibodies were immobilized in specific spots on coated glass slides to target and capture the corresponding cytokines. A cocktail of biotinylated antibodies was added to probe the bound cytokines. A biotin-streptavidin complex conjugated with fluorescent dye, used to visualize the fluorescence signals, and was detected with a GenePix 4000B system (Axon Instruments, USA). GenePix Pro 6.0 software (Axon Instruments, USA) was used for densitometric analysis of the spots.

**Statistical analysis**

All data were based on the fluorescence intensities after removing background image. Then background-subtracted raw data of the 37 cytokines were normalized by median centering and analyzed statistically by SPSS (version 20.0). Differences in categorical
variables were examined using a Chi-squared ($\chi^2$) test. Numerical variables were denoted by means ± standard deviation (SD) or by medians with minimum to maximum range. Unpaired continuous variables were compared by Student’s t-tests or the Mann-Whitney U test. Correlations between clinical characteristic and expression of cytokines, as well as correlations between cytokines, were performed using the Pearson’s test or Spearman’s test. Statistical significance was determined if $p < 0.05$.

Results

- General patient data are presented in Table 1. Six POAG patients (male: female=4:2) were enrolled in case group with a mean age of 56 ± 10.9 years and seven age-related cataract patients (male: female=3:4) were enrolled in the control group (61.6 ± 5.3 years of age). Age, sex, IOP, and BCVA did not have significant differences between POAG and control groups.

Cytokines antibody array

- Data regarding the signal intensity of the cytokines are shown in Table 2. Expression of sVEGFR-1 in the aqueous humor of POAG patients is higher up to 1.65-fold, which was a significant difference when compared with the control group (signal intensities: 550.8 ± 126.3 and 334.4 ± 121.1, respectively; Student’s test: $p = 0.009$). There was a marked greater of vWF levels in the aqueous humor of POAG patients compared with control group, as demonstrated in Figure 1 (1.89 fold; 197 with range from 85 to 372; 104 with range from 81 to 166, respectively; Mann-Whitney U test: $p = 0.035$). Expression of angiopoietin-2 in POAG group showed a 2.0-fold increase compared to the control group.
(signal intensities: 500 with range from 296 to 1638; 250 with range from 171 to 429, respectively; Mann-Whitney U test; p = 0.022). Similarly, TNF-α expression was higher in the POAG group with statistical significance (signal intensities: 404.5 with range from 377 to 440; 359 with range from 335 to 430, respectively; Mann-Whitney U test; p = 0.014). No statistically significant differences were seen in the other cytokines between POAG and control groups.

**Correlation analysis**

Linear correlation analysis revealed that expression levels of sVEGFR-1, VEGF-A, and PAI-1 in aqueous humor were significant correlated with IOP levels (r = 0.647, 0.602, and -0.593, respectively; p < 0.05), as shown in Table 3. Cytokines vWF, TNF-α, and angiopoietin-1 showed a marked positive linear relationship to sVEGFR-1 (r = 0.646, 0.824, and 0.582, respectively; p < 0.05). In addition to sVEGFR-1, TNF-α, angiopoietin-1 and CCL-2/MCP-1 were also positively correlated with vWF (r = 0.569, 0.597, and 0.726, respectively; p < 0.05). TNF-α showed a positive correlation with angiopoietin-2 (r = 0.555, p = 0.049). In POAG patients, the signal strength of MMP-1 (r = -0.936, p = 0.006) and Fas-L (r = -0.681, p = 0.03) demonstrated a strong negative correlation with values of mean deficit (MD), which is obtained from visual field testing.

**Discussion**

Results of the present study show that in the aqueous humor of POAG patients, cytokines involved in vascular endothelial function, including sVEGFR-1, vWF, and angiopoietin-2, as well as in inflammatory reactions, including TNF-α, are significantly greater compared with control patients. Therefore, there is a potential association between POAG pathogenesis and vascular endothelial dysfunction as well as inflammation.
Since vWF is synthesized by endothelial cells and released when endothelial cell are injured, it is a good marker of endothelial dysfunction [13,14]. In ocular diseases, vWF is highly expressed in vitreous and serum of diabetes retinopathy [15]. In glaucoma patients, vWF levels are shown to be high in plasma (by ELISA) [16] and iris vasculature (by immunohistochemistry) [17] compared to control groups. However, considering that endothelial cells are distributed over the whole body, the plasma level could be easily influenced by systemic conditions. Additionally, vWF is a kind of secreted cytokines; therefore, it is hard to reflect its true level using immunohistochemistry in real time. By investigating vWF levels in the aqueous humor, our study showed high levels in POAG patients, which is in accordance with previous results and supports the hypothesis that endothelium dysfunction is involved in POAG pathogenesis.

VEGF is a vasoactive cytokine produced by endothelial cells, epithelial cells, pericytes, astrocytes, Müller cells and tumor cells [18]. Higher levels of VEGF are thought to be a response to active angiogenesis in some diseases and could be observed in physiological repair processes [19,20]. Animal model studies as well as clinical patient research show that VEGF levels increased in certain intraocular diseases with active intraocular neovascularization including retinopathy of prematurity, central retinal vein occlusion, branch retinal vein occlusion, proliferative diabetic retinopathy, and neovascular glaucoma [7,20-22]. A previous study showed the VEGF levels in the plasma were significantly elevated both in POAG and normal tension glaucoma patients when compared to cataract patients [16]. Our present array study reveals no significant difference in VEGF levels (VEGF-A, VEGF-C and VEGF-D) between POAG aqueous humor and control patients, which is consistent with a previous study [8], though the result is in opposition to the
conclusions from other studies [23]. Using a sandwich ELISA kit, another study demonstrated a statistically significant increase in aqueous levels of VEGF in both POAG and primary angle closure glaucoma patients when compared to cataract patients [9]. The difference may be influenced by the assessment methods used in our research and in the previous study, namely using an array-based multiplex sandwich ELISA system and not the traditional ELISA method.

Splicing the mRNA that can be translated into VEGF receptor-1 produces sVEGFR-1, a soluble form of VEGFR-1. VEGFR-1 is expressed mainly on the surface of vascular endothelial cells and is one of the main VEGF receptors; it regulates and combines with VEGF to recruit monocytes and macrophages and influences vascular permeability involved in angiogenesis and inflammation. Unlike VEGFR-1, soluble receptors act as endogenous antagonists that bind to VEGF-A to exert an anti-hemangiogenic effect and impair the vasodilatory response [18]. In some ocular diseases related to ischemia, sVEGFR-1 was reported to increased, such as in cases of diabetic retinopathy [24] and retinal vein occlusion [7,22]. Until now, changes in sVEGFR-1 levels in glaucomatous eyes have not been reported. Only one previous study reported that in the plasma of POAG patients, sVEGFR-1 expression levels were lower than that in the control group [16], which was not in accordance with our present result. There may be several reasons for this discrepancy. First, the test methods were different: the previous study used a traditional ELISA while our group utilized an antibody-based cytokine array; different principles, sensitivity and analytical methods may result in variance. Furthermore, quite different from aqueous humor, plasma is exposed to systemic circumstances and do not reflect the ocular condition with the same accuracy. Moreover, the blood ocular barrier is a special physiological structure that can intensify this variation.
Angiopoietin-2 is a crucial regulator of angiogenesis and vascular homeostasis, exerting an antagonistic effect for angiopoietin-Tie2 signaling [25]. In patients with age-related macular degeneration, angiopoietin-2 is upregulated and correlates with central macular thickness [26]. Moreover, angiopoietin-2 is reported to be essential for the formation and integrity of Schlemm’s canal and in turn stabilizing the IOP: a mice experiment showed that the loss of angiopoietin-Tie2 signaling severely impaired Schlemm’s canal integrity, resulting in elevated IOP and retinal neuron damage [27]. In our present results, the expression of angiopoietin-2 was remarkably higher in POAG patients, also suggesting that there is an impairment of angiopoietin-Tie2 signaling damaging Schlemm’s canal which may underly POAG pathogenesis.

Additionally, TNF-α as a cytokine causing cell death and involving inflammation, has proven to be increased in glaucomatous aqueous humor [8,28] and was also elevated in POAG patients in our study. Other inflammatory cytokines in this study, including CCL2/MCP-1, CCL3/MIP-1α, CCL4/MIP-1β, CCL5/RANTES, IL-1b, IL-6, IL-8, IL-12 showed no difference between control and glaucoma groups, in accordance with previous study [8,29].

This study has a few limitations. First, it is a case-control study with a relatively small number of participants. Consequently, our study cannot determine causality. In addition, POAG patients had accepted treatments before surgery, which is another difference from control group patients. Nonetheless, patients with age-related cataracts may have other unaccounted differences from healthy people. We implemented our inclusion and exclusion criteria to select the most appropriate non-age-related cataract patients for our
control group.

In conclusion, the present study profiled vascular and inflammatory cytokines in the aqueous humor of POAG patients and showed the associations between POAG and cytokine changes. Furthermore, we first found the elevated concentrations of vWF, sVEGFR-1, and angiopoietin-2 in glaucomatous aqueous humor. The elevated cytokines are consistent with the hypothesis that vascular and inflammatory factors are related to POAG pathogenesis. Further research is needed to clarify the role of cytokines and to understand the altered micro-environment in the anterior chamber.

Declarations

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CONFLICTS OF INTEREST

The authors declare that they have no conflict of interest.

ETHICS APPROVAL
All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards.

References

1. Quigley HA (1996) Number of people with glaucoma worldwide. Br J Ophthalmol 80 (5):389-393

2. Riva CE, Salgarello T, Logean E, Colotto A, Galan EM, Falsini B (2004) Flicker-evoked response measured at the optic disc rim is reduced in ocular hypertension and early glaucoma. Invest Ophthalmol Vis Sci 45 (10):3662-3668. 'doi:'10.1167/iovs.04-0100

3. Grieshaber MC, Mozaffarieh M, Flammer J (2007) What is the link between vascular dysregulation and glaucoma? SURV OPHTHALMOL 52 Suppl 2:S144-S154. 'doi:'10.1016/j.survophthal.2007.08.010

4. Babapoor-Farrokhran S, Jee K, Puchner B, Hassan SJ, Xin X, Rodrigues M, Kashiwabuchi F, Ma T, Hu K, Deshpande M, Daoud Y, Solomon S, Wenick A, Lutty GA, Semenza GL, Montaner S, Sodhi A (2015) Angiopoietin-like 4 is a potent angiogenic factor and a novel therapeutic target for patients with proliferative diabetic retinopathy. Proceedings of the National Academy of Sciences 112 (23):E3030-E3039. 'doi:'10.1073/pnas.1423765112

5. Noma H, Funatsu H, Mimura T (2012) Vascular endothelial growth factor and interleukin-6 are correlated with serous retinal detachment in central retinal vein occlusion. CURR EYE RES 37 (1):62-67. 'doi:'10.3109/02713683.2011.614370

6. Zhu X, Wolff D, Zhang K, He W, Sun X, Lu Y, Zhou P (2015) Molecular Inflammation in the Contralateral Eye After Cataract Surgery in the First Eye. Investigative Ophthalmology & Visual Science 56 (9):5566. 'doi:'10.1167/iovs.15-16531

7. Noma H, Mimura T, Yasuda K, Shimura M (2015) Role of Soluble Vascular Endothelial
Growth Factor Receptor Signaling and Other Factors or Cytokines in Central Retinal Vein Occlusion With Macular Edema.

INVEST OPHTH VIS SCI 56 (2):1122-1128.
'doi:'10.1167/iovs.14-15789

8. Chua J, Vania M, Cheung CM, Ang M, Chee SP, Yang H, Li J, Wong TT (2012) Expression profile of inflammatory cytokines in aqueous from glaucomatous eyes. MOL VIS 18:431-438

9. Hu DN, Ritch R, Liebmann J, Liu Y, Cheng B, Hu MS (2002) Vascular endothelial growth factor is increased in aqueous humor of glaucomatous eyes. J GLAUCOMA 11 (5):406-410

10. Kuchtey J, Rezaei KA, Jaru-Ampornpan P, Sternberg PJ, Kuchtey RW (2010) Multiplex cytokine analysis reveals elevated concentration of interleukin-8 in glaucomatous aqueous humor. Invest Ophthalmol Vis Sci 51 (12):6441-6447. 'doi:'10.1167/iovs.10-5216

11. Izzotti A, Longobardi M, Cartiglia C, Saccà SC (2010) Proteome Alterations in Primary Open Angle Glaucoma Aqueous Humor. J PROTEOME RES 9 (9):4831-4838.
'doi:'10.1021/pr1005372

12. Sacca SC, Centofanti M, Izzotti A (2012) New proteins as vascular biomarkers in primary open angle glaucomatous aqueous humor. Invest Ophthalmol Vis Sci 53 (7):4242-4253. 'doi:'10.1167/iovs.11-8902

13. Lip GY, Blann A (1997) von Willebrand factor: a marker of endothelial dysfunction in vascular disorders? CARDIOVASC RES 34 (2):255-265

14. Randi AM, Smith KE, Castaman G (2018) von Willebrand factor regulation of blood vessel formation. BLOOD 132 (2):132-140. 'doi:'10.1182/blood-2018-01-769018

15. Adamiec-Mroczek J, Oficjalska-Mlynczak J, Misiuk-Hojlo M (2009) Proliferative diabetic retinopathy-The influence of diabetes control on the activation of the intraocular
molecule system. Diabetes Res Clin Pract 84 (1):46-50. ‘doi:’10.1016/j.diabres.2009.01.012

16. Lip PL, Felmeden DC, Blann AD, Matheou N, Thakur S, Cunliffe IA, Lip GY (2002) Plasma vascular endothelial growth factor, soluble VEGF receptor FLT-1, and von Willebrand factor in glaucoma. Br J Ophthalmol 86 (11):1299-1302

17. Wojnicz R (2014) von Willebrand factor in iris vasculature of glaucoma patients. MED SCI MONITOR 20:1051-1055. ‘doi:’10.12659/MSM.890176

18. LeCouter J, Gerber H, Ferrara N (2003) The biology of VEGF and its receptors 9 (6):669-676. ‘doi:’10.1038/nm0603-669

19. Stockhammer G, Obwegeser A, Kostron H, Schumacher P, Muigg A, Felber S, Maier H, Slavc I, Gunsilius E, Gastl G (2000) Vascular endothelial growth factor (VEGF) is elevated in brain tumor cysts and correlates with tumor progression. ACTA NEUROPATHOL 100 (1):101-105

20. Leung DW, Cachianes G, Kuang WJ, Goeddel DV, Ferrara N (1989) Vascular endothelial growth factor is a secreted angiogenic mitogen. SCIENCE 246 (4935):1306-1309

21. Noma H, Mimura T, Yasuda K, Shimura M (2014) Vascular Endothelial Growth Factor and Its Soluble Receptors-1 and -2 in Iris Neovascularization and Neovascular Glaucoma. OPHTHALMOLOGICA 232 (2):102-109. ‘doi:’10.1159/000360303

22. Noma H, Mimura T, Yasuda K, Shimura M (2014) Role of soluble vascular endothelial growth factor receptors-1 and -2, their ligands, and other factors in branch retinal vein occlusion with macular edema. Invest Ophthalmol Vis Sci 55 (6):3878-3885. ‘doi:’10.1167/iovs.14-13961

23. Li Z, Van Bergen T, Van de Veire S, Van de Vel I, Moreau H, Dewerchin M, Maudgal PC, Zeyen T, Spileers W, Moons L, Stalmans I (2009) Inhibition of Vascular Endothelial Growth Factor Reduces Scar Formation after Glaucoma Filtration
Surgery. Investigative Ophthalmology & Visual Science 50 (11):5217.  'doi:'10.1167/iovs.08-2662

24. Javanmard SH, Hasanpour Z, Abbaspoor Z, Naderian GA, Jahanmard M (2012) Aqueous concentrations of VEGF and soluble VEGF receptor-1 in diabetic retinopathy patients. J RES MED SCI 17 (12):1124-1127

25. Augustin HG, Young Koh G, Thurston G, Alitalo K (2009) Control of vascular morphogenesis and homeostasis through the angiopoietin-Tie system. NAT REV MOL CELL BIO 10 (3):165-177. 'doi:'10.1038/nrm2639

26. Ng DS, YipYW, Bakthavatsalam M, Chen LJ, Ng TK, Lai TY, Pang CP, Brelen ME (2017) Elevated angiopoietin 2 in aqueous of patients with neovascular age related macular degeneration correlates with disease severity at presentation. Sci Rep 7:45081. 'doi:'10.1038/srep45081

27. Kim J, Park D, Bae H, Park DY, Kim D, Lee C, Song S, Chung T, Lim DH, Kubota Y, Hong Y, He Y, Augustin HG, Oliver G, Koh GY (2017) Impaired angiopoietin/Tie2 signaling compromises Schlemm’s canal integrity and induces glaucoma. J CLIN INVEST 127 (10):3877-3896. 'doi:'10.1172/JCI94668

28. Sawada H, Fukuchi T, Tanaka T, Abe H (2010) Tumor necrosis factor-alpha concentrations in the aqueous humor of patients with glaucoma. Invest Ophthalmol Vis Sci 51 (2):903-906. 'doi:'10.1167/iovs.09-4247

29. Kokubun T, Tsuda S, Kunikata H, Yasuda M, Himori N, Kunimitsu-Sanuki S, Maruyama K, Nakazawa T (2017) Characteristic Profiles of Inflammatory Cytokines in the Aqueous Humor of Glaucomatous Eyes. OCUL IMMUNOL INFLAMM 26 (8):1177-1188. 'doi:'10.1080/09273948.2017.1327605

Tables

Table 1. Demographic and clinical data of POAG and cataract patients.
|                  | Number | Controls | P value |
|------------------|--------|----------|---------|
| **Age (years, Mean ±SD)** | 56.0±10.9 | 61.6±5.3 | 0.2     |
| **Gender (male/female)** | 4/2     | 3/4      | 0.5     |
| **IOP (Mean ±SD)**   | 23.5±14.3 | 14.9±2.6 | 0.2     |
| **BCVA (Median, min-max)** | 0.32 (0.04-1.00) | 0.40 (0.02-0.50) | 0.5     |

Abbreviations: SD, standard deviation; IOP, intraocular pressure; POAG, primary open-angle glaucoma; BCVA, best corrected visual acuity. * Fisher's Exact χ² Test; # Independent-sample Mann-Whitney U Test

Table 2. Signal intensity of aqueous cytokines with cytokines antibody array.
Vascular related cytokines

| Vascular related cytokines | Ratio: POAG/Control | Control | POAG | P value |
|----------------------------|---------------------|---------|------|---------|
| sVEGF-R1 **                | 1.65                | 334.4 ±121.1 | 550.8 ±126.3 | 0.009   |
| vWF *                     | 1.89                | 104 (81-166) | 197 (85-372) | 0.035   |
| Angiopoietin-2 *          | 2.00                | 250 (171-429) | 500 (296-1638) | 0.022   |
| Endothelin-1              | 1.11                | 185 (167-251) | 204.5 (180-735) | 0.37    |
| VEGF-A                    | 0.95                | 594.4 ±147.0 | 565.3 ±327.8 | 0.84    |
| VEGF-C                    | 0.81                | 39.4 ±11.4 | 32.0 ±8.0 | 0.21    |
| VEGF-D                    | 1.08                | 46.6 ±14.7 | 50.2 ±25.7 | 0.76    |
| VEGF-R3                   | 0.78                | 58.6 ±14.5 | 45.5 ±17.4 | 0.17    |
| PIGF                      | 0.94                | 35.4 ±15.5 | 33.3 ±16.1 | 0.82    |
| PDGF-AB                   | 0.81                | 54.6 ±23.4 | 44.3 ±9.9 | 0.34    |
| PDGF-BB                   | 0.71                | 42.4 ±15.9 | 30.2 ±14.3 | 0.17    |
| SDF-1α                    | 0.82                | 64.1 ±22.4 | 52.5 ±15.1 | 0.31    |
| SDF-1β                    | 1.00                | 63 (35-80) | 63 (53-113) | 0.53    |
| Angiopoietin-1            | 1.50                | 140 (94-199) | 209.5 (122-672) | 0.10    |
| Angiostatin               | 0.84                | 119 (90-176) | 100.5 (89-253) | 0.63    |
| ANGPTL-4                  | 1.06                | 52 (39-57) | 55 (29-82) | 0.73    |
| Tie-1                     | 1.10                | 115 (84-125) | 126.5 (96-216) | 0.30    |
| Tie-2                     | 0.92                | 79 (55-93) | 72.5 (41-200) | 0.84    |
| EPO                       | 1.02                | 152.1 ±28.1 | 154.5 ±26.1 | 0.88    |

Data were shown in mean ± standard deviation or median with range from minimum to maximum. p-values were obtained from Student’s t test or Mann–Whitney U test (significant p-values are highlighted in bold). All data of cytokines were showed by signal intensity. *p<0.05; **p<0.01. POAG, primary open-angle glaucoma

Table 3. Correlations among all cytokines.

|       | sVEGFR-1 | vWF |
|-------|----------|-----|
| IOP   | sVEGFR-1 |     |
| r     | 0.647*   |     |
| p value | 0.02     |    |
| sVEGFR-1 | 1.00     |   |
| p value |          |    |
| vWF    | 0.646    | 0.0 |

18
|                |        |        |        |      |      |      |
|----------------|--------|--------|--------|------|------|------|
| vWF            | 0.32   | 0.31   | 0.646* | 0.02 | 1.00 | -    |
| TNF-α          | 0.32   | 0.31   | 0.824**| <0.001| 0.569*| 0.0  |
| Angiopoietin-2 | 0.15   | 0.65   | 0.47   | 0.11 | 0.39 | 0.1  |
| Endothelin-1   | 0.10   | 0.75   | 0.46   | 0.12 | 0.54 | 0.0  |
| VEGF-A         | 0.602* | 0.04   | 0.29   | 0.34 | -0.01| 0.9  |
| VEGF-C         | -0.04  | 0.90   | -0.14  | 0.65 | -0.35| 0.2  |
| VEGF-D         | 0.34   | 0.28   | 0.12   | 0.71 | -0.09| 0.7  |
| VEGFR-3        | 0.14   | 0.66   | -0.18  | 0.55 | -0.30| 0.3  |
| PIGF           | 0.48   | 0.11   | 0.41   | 0.16 | 0.29 | 0.3  |
| PDGF-AB        | -0.03  | 0.94   | 0.08   | 0.79 | -0.02| 0.9  |
| PDGF-BB        | 0.30   | 0.35   | 0.02   | 0.96 | -0.11| 0.7  |
| SDF-1α         | 0.07   | 0.82   | 0.07   | 0.83 | -0.10| 0.7  |
| SDF-1β         | 0.31   | 0.32   | 0.42   | 0.16 | 0.30 | 0.3  |
| Angiopoietin-1 | -0.28  | 0.38   | 0.582* | 0.04 | 0.597*| 0.0  |
| Angiostatin    | 0.13   | 0.69   | 0.18   | 0.56 | 0.34 | 0.2  |
| ANGPTL-4       | 0.41   | 0.19   | 0.49   | 0.09 | 0.34 | 0.2  |
| Tie-1          | 0.40   | 0.19   | 0.22   | 0.47 | 0.21 | 0.4  |
| Tie-2          | -0.03  | 0.92   | 0.04   | 0.89 | 0.40 | 0.1  |
| MMP-1          | -0.34  | 0.28   | -0.32  | 0.28 | -0.41| 0.1  |
| MMP-9          | -0.50  | 0.10   | -0.09  | 0.78 | 0.07 | 0.8  |
| PAI-1          | -0.593*| 0.04   | 0.15   | 0.63 | 0.30 | 0.3  |
| EPO            | 0.17   | 0.60   | 0.18   | 0.55 | -0.03| 0.9  |
| ICAM-1         | 0.17   | 0.61   | 0.00   | 1.00 | -0.09| 0.7  |
| Fas            | 0.38   | 0.22   | 0.27   | 0.38 | 0.32 | 0.2  |
| Fas L          | -0.16  | 0.62   | -0.29  | 0.34 | -0.14| 0.6  |
| IL-6           | 0.07   | 0.82   | 0.10   | 0.73 | 0.13 | 0.6  |
| IL-1b          | 0.40   | 0.20   | 0.13   | 0.67 | 0.01 | 0.9  |
| IL-8           | 0.23   | 0.47   | 0.39   | 0.19 | 0.01 | 0.9  |
| Protein       | Correlation Coefficient | p Value |
|--------------|-------------------------|---------|
| IL-12p70     | -0.18                   | 0.57    |
| CCL-2/MCP-1  | 0.44                    | 0.16    |
| CCL-3/MIP-1α | 0.26                    | 0.42    |
| CCL-4/MIP-1β | -0.43                   | 0.16    |
| CCL-5/RANTES | 0.28                    | 0.38    |
| TIMP-1       | 0.32                    | 0.31    |
| TIMP-2       | 0.32                    | 0.30    |
| Endoglin     | 0.28                    | 0.37    |

Correlation coefficient and p values are calculated by Pearson’s correlation or Spearman’s correlation. r, correlation coefficient α, non-parametric statistics by Spearman’s correlation. *P<0.05; **P<0.01.

Figures
Results of cytokine antibody arrays. Representative results for the expression levels of vWF (box d), TNF-α (box e), angiopoietin-2 (box f), and sVEGFR-1 (box g) in glaucoma eyes (a) and control eyes (b). Expression levels of sVEGFR-1, vWF, TNF-α, and angiopoietin-2 in glaucoma eyes and control eyes (c). The line of sVEGFR-1 represents the mean value with the standard error of measurement and the lines of the others represent the median value. *p < 0.05; **p < 0.01. sVEGFR-1, soluble vascular endothelial growth factor receptor 1; vWF, von Willebrand factor; TNF-α, tumor necrosis factor.