Aqueous Humor Biomarkers of Retinal Glial Cell Activation in Patients With or Without Age-Related Cataracts and With Different Stages of Diabetic Retinopathy

Minjie Zhao,1 Shuzhi Zhao,2–6 Min Tang,2–6 Tao Sun,2–7 Zhi Zheng,2–6 and Mingming Ma2–6

1Department of Ophthalmology, Yixing People’s Hospital, Jiangsu University, Zhenjiang, China
2Department of Ophthalmology, Shanghai General Hospital, Shanghai, China
3National Clinical Research Center for Eye Diseases, Shanghai, China
4Shanghai Key Laboratory of Ocular Fundus Diseases, Shanghai, China
5Shanghai Engineering Center for Visual Science and Photomedicine, Shanghai, China
6Shanghai Engineering Center for Precise Diagnosis and Treatment of Eye Diseases, Shanghai, China
7Shanghai Eye Diseases Prevention & Treatment Center/Shanghai Eye Hospital, Shanghai, China

Correspondence: Mingming Ma, No. 85, Wujin Road, Hongkou District, Shanghai, China; mingming.ma@shgh.cn.
Zhi Zheng, No. 85, Wujin Road, Hongkou District, Shanghai, China; zzheng88@sjtu.edu.cn.
Tao Sun, 85 Wujin Road, Hongkou District, Shanghai, China; drsuntao@yeah.net.
MZ, SZ, and MT have contributed equally to this work.

RESULTS. We enrolled 82 patients. The AQP1 concentration was higher in AH from cataract control patients than in control patients without cataracts (P < 0.05). The AQP1 concentration was also higher in patients with DM, NPDR, and PDR than in controls (P < 0.05). The concentrations of AQP4 and GFAP were significantly increased in patients with NPDR and PDR (P < 0.05) but not in patients with DM. Kir4.1 concentration was significantly decreased in patients with NPDR and PDR (P < 0.05), but the decrease in patients with DM did not reach significance. There were no differences in AQP4, Kir4.1, and GFAP between patients with and without ARCs.

CONCLUSIONS. Increased AQP1 in AH may be a biomarker for ARCs in patients without diabetes and a biomarker for retinal glial cell activation in patients with diabetes without cataracts. AQP4, Kir4.1, and GFAP levels in AH suggested that retinal glial cell activation was affected by the progression of DR.

Keywords: retinal glial cell, diabetic retinopathy, age-related cataract, aqueous humor

The activation of retinal glial cells in DR can be monitored by testing the aqueous humor (AH). The AH is produced by the nonpigmented ciliary body epithelium, the protein and ion content is different from that of the plasma, and it continuously exchanges substances with tissues through direct and indirect contact. The levels of some inflammatory and angiogenic cytokines are changed in the AH of patients with DR. Indeed, the levels of certain proteins in the AH can be used to indicate the status of eye tissues and serve as biomarkers to predict the development of DR or DM. Previous studies have found that the activation of Müller cells was accompanied by increases of water channel aquaporin 1 (AQP1), water channel aquaporin 4 (AQP4), and glial fibrillary acidic protein (GFAP) in the AH and that changes of the proteins could be used as biological markers for the activation of Müller cells.

Diabetic retinopathy (DR) is the leading cause of blindness in working-age people in developed countries and is the most common chronic microvascular complication of diabetes. The prevalence of DR in diabetes mellitus (DM) is estimated as 44.9% in China. Although the diagnosis of DR is based on vascular abnormality, retinal neovascularization has been detected in the early stage of DM before observable vascularopathy. Activation of retinal glial cells has been seen in early stage of DM, with the release of cytokines such as VEGF, TNF-α, IL-1β, and others, which results in increased vascular permeability. Retinal glial cell activation at an early stage may have a compensatory protective effect on the retina, but long-term activation may accelerate the progression of DR. Changes in retinal glial cells are thought to have a role in the development of DR.
However, based on the published studies, there are still some questions that need to be clarified. First, APQ1 is normally expressed not only in the retina but also in the lens epithelium. Its expression is increased in cataract lens epithelium. The expression of AQP in the AH was found to be influenced by anterior segment disease. Therefore, it is necessary to determine the differences of AQP1 present in the AH of those with and without cataracts. However, there have been no studies of AQP1 in the AH of patients with cataract. Second, AQP1, AQP4, and GFAP expression in the retina was found to be influenced by hypertension in animal experiments. Although a previous study found that AQP1, AQP4, and GFAP protein expression in the AH changed in DR, the influence of hypertension on the findings could not be completely excluded. Therefore, further study may be needed to achieve a more comprehensive, in-depth understanding of the performance of glial cell activation in the AH and to exclude the influence of hypertension. Finally, there have been no studies of the expression of AQP1, AQP4, Kir4.1, and GFAP at different stages of DR. Answering the questions may help us to further understand the mechanism of DR. Therefore, the purpose of this study was to investigate the changes in the content of AQP1, AQP4, GFAP, and Kir4.1 in AH at different stages of DR as well as the influence of age-related cataracts (ARCs) on these indicators.

MATERIALS AND METHODS

Study Design

This study was a cross-sectional, case-control series evaluation. Patients with and without ARCs were selected to investigate the influence of ARCs on the expression of AQP1, AQP4, Kir4.1, and GFAP in the AH. Furthermore, in order to clarify the trends of these factors in the progression of DR, patients without diabetes, diabetes patients without DR, NPDR patients and PDR patients were included in the groups formed by stratification by ARC status. Each participant underwent a complete ophthalmologic examination before surgery, including best-corrected visual acuity (BCVA), intraocular pressure (IOP), slit-lamp examination, color fundus photos, and optical coherence tomography. Fluorescein fundus angiography was performed in the patients with DM. The presence of DR was graded by internationally established color fundus photos criteria. Blood pressure, fasting blood glucose (FBG), and hemoglobin A1c (HbA1c) were checked on the morning of operation. The history of medication use was also collected.

Study Population

All 82 patients who had eye surgery at the Department of Ophthalmology, Shanghai First People’s Hospital between February 2019 and August 2020 were selected. Only patients with ARCs were included in the cataract group. The lens was examined after pupillary dilation with tropicamide (1%) and phenylephrine (2.5%). Two ophthalmologists determined the cataract with the aid of a slit-lamp biomicroscope. The patients with cataract included in the study were stages NC2 to NC4, C2 to C4, and P2P3 according to the Lens Opacities Classification System III criteria. The patients were divided into eight groups. Four groups included patients with ARCs (cataract group) and four did not (no-cataract group). Each of the groups included four other study groups that included patients with DM, NPDR, PDR, and controls. The group assignments of the 82 patients and controls are shown in the flow diagram in Figure 1.

The inclusion criteria were an indication for eye surgery and agreement to allow extraction of AH. The main exclusion criteria were receipt of any other treatments (laser, intravitreal injections of anti-VEGF drugs); vitreous hemorrhage; a history of eye surgery; previous diagnosis of glaucoma, ocular hypertension, or concomitant retinal disease; presence of any neurodegenerative disorders (Parkinson, Alzheimer, etc.); or history of hypertension. Informed consent was obtained for each patient. The study was performed following the ethical principles of the Declaration of Helsinki regarding experimentation involving humans. The local ethics committee approved the study.

AH Collection, Storage, and Total Protein Assay

Patients with pterygium excision or intravitreal injection surgery underwent standard preoperative procedures used for cataract surgery. The skin around the eyes was disinfected with 5% povidone iodine. After topical anesthesia with bupivacaine hydrochloride, 5% povidone iodine was used to disinfect the conjunctival sac before washing with an abundance of normal saline. AH (150–200 μL) was aspirated from the anterior chamber with microscope guidance using...
a 30-gauge needle and an insulin syringe. The AH samples were transferred to three labeled microfuge tubes containing a protease inhibitor and stored at −80°C until analysis. The protein concentration was assayed by a standard bovine serum albumin method.

ELISAs for AQP1, AQP4, Kir4.1, and GFAP

The ELISA kits (with their ranges and lower limits) used for the protein assays included GFAP (HM10951; Bioswamp, Hubai, China; 0.125–10 ng/mL, <0.025 ng/mL), Kir4.1 (MBS2707202; MyBioSource, Inc., San Diego, CA, USA; 1.56–100 ng/mL, <0.58 ng/mL), AQP1 (xy-H0487C; Xin Yu Biotech Co., Ltd., Shanghai, China; 5–80 ng/L, <1.2 ng/L), and AQP4 (xy-H0490c; Xin Yu Biotech Co., Ltd.; 5–120 ng/L, <1.8 ng/L), and all were used following the manufacturers’ respective protocols. The colorimetric signal was quantified using an ELISA reader (Freedom EVO; Tecan Group Ltd., Männedorf, Switzerland) at 490 to 560 nm. The optical density data were normalized to total protein as assessed at the beginning. The GFAP, Kir4.1, AQP1, and AQP4 concentrations were calculated with reference to linearized standard curves obtained following completion of the ELISA assays. Duplicate measurements were obtained for all samples.

Statistical Analysis

The AQP1, AQP4, GFAP, and Kir4.1 results were expressed as mean ± SD. ANOVA followed by Tukey-Kramer post hoc analysis was used to compare the results of the groups with and without cataracts. An independent sample t test was used to test between-group differences in patients with cataract and no cataract. Statistical analysis was performed with SPSS version 17.0 for Windows (SPSS, Chicago, IL, USA). A P value of <0.05 was considered to indicate a statistically significant difference.

Results

Population

The patient characteristics are presented in the Table 1. In patients without cataracts, there were significant differences in BCVA, DM duration, FBG, and HbA1c in the control, DM, NPDR, and PDR groups (all P < 0.001). There was no significant difference in mean age (P = 0.12), IOP (P = 0.79), and blood pressure (P > 0.05) among the four groups. In those with cataracts, there was no significant difference in mean IOP (P = 0.81) and blood pressure (P > 0.05) among the control, DM, NPDR, and PDR groups. There was a significant difference in mean age (P = 0.017), DM duration (P < 0.001), BCVA (P < 0.001), FBG (P < 0.001), and HbA1c (P < 0.001) among the four groups. Histories of antihypertensive medications were not available. In patients with diabetes, insulin and/or oral hypoglycemic drugs, such as metformin, sulfonfonylurea, or α-glucosidase inhibitor, were used to control blood glucose.

Influence of Cataracts on AH AQP1 Level

The results of AQP1 concentration in AH are shown in Table 2. There were significant differences in the concentration of AQP1 in the AH of patients in the control, DM, NPDR, and PDR groups both with and without cataracts (P < 0.001) and with and without cataracts (P < 0.001) and with
catacrats (P < 0.001). The difference in the AQP1 concentration in the AH of the no-cataract control and the cataract control groups was significant (P < 0.001; Fig. 2A).

In patients in the cataract and no-cataract groups, significant increases in the AQP1 concentration in the AH were found in the DM, NPDR, and PDR groups compared with the control group (all P < 0.001). Significant increases were also found in the NPDR and PDR groups compared with the DM group (both P < 0.001). The difference of the AQP1 concentrations in the PDR and NPDR groups was significant (P < 0.001; Fig. 2A).

### AQP4 in the AH of Patients at Different Stages of DR

As shown in Table 2, there were significant differences in the concentration of AQP4 in the AH among the control, DM, NPDR, and PDR groups within the no-cataract (P < 0.001) and the cataract (P < 0.001) groups. The difference of the AQP4 concentration in the AH in the no-cataract control and cataract control groups was not significant (P > 0.05; Fig. 2B).

In patients in the cataract and no-cataract groups, significant increases in AQP4 concentration in the AH were found in the NPDR and PDR groups compared with the control group (both P < 0.001). Significant increases were also found in the NPDR and PDR groups compared with the DM group (both P < 0.001). Furthermore, the difference of AQP4 in the PDR and NPDR groups was significant (P < 0.001; Fig. 2B).

### Kir4.1 in the AH of Patients at Different Stages of DR

As shown in Table 2, there were significant differences in the concentration of Kir4.1 among the control, DM, NPDR, and PDR groups in the no-cataract (P < 0.001) and cataract (P < 0.001) groups. The difference of the Kir4.1 concentration in the AH between the no-cataract control and cataract control groups was not significant (P > 0.05).

In patients in the cataract and no-cataract groups, significant decreases in Kir4.1 concentration in the AH were found in NPDR and PDR groups compared with the control group (both P < 0.001). Significant decreases were also found in the NPDR and PDR groups compared with the DM group (both P < 0.001). Furthermore, the difference of Kir4.1 in the PDR and NPDR groups was significant (P < 0.001; Fig. 2C).

### GFAP in the AH of Patients at Different Stages of DR

As shown in Table 2, there were significant differences in GFAP concentration in the AH among the control, DM, NPDR, and PDR groups in the no-cataract (P < 0.001) and cataract (P < 0.001) patients. The difference of GFAP concentration in the no-cataract control and cataract control groups was not significant (P > 0.05).

In patients in the cataract and no-cataract groups, significant increases in GFAP concentration in the AH were found in the NPDR and PDR groups compared with the control group (both P < 0.001). Significant increases were also found in the NPDR and PDR groups compared with the DM group (both P < 0.001). Furthermore, the difference
of GFAP in the PDR and NPDR groups was significant (P < 0.001; Fig. 2D).

**DISCUSSION**

To our knowledge, this is the first study to report the influence of cataracts on the AQP1 in the AH and the changes of AQP1, AQP4, GFAP, and Kir4.1 in the AH at different stages of DR. The increased concentration of AQP1 in the AH indicated that it may not be suitable as a marker of retinal glial cell activation in ARC subjects reported in the previous study. Differences in the expression of AQP1, AQP4, GFAP, and Kir4.1 in the AH that were found at different stages of DR indicated that retinal glial cell activation was affected by the progression of DR. We also found that the changes in the AQP4 and Kir 4.1 concentrations in the AH at different stages of DR were not the same as previously reported.

AQP1 is an important water channel in subpopulations of amacrine and photoreceptor cells in normal retina and in lens epithelium. In animal models of streptozotocin-induced DM, the water channel in the Müller cells that surround the superficial retinal vessels switches from AQP4 to AQP1 at an early stage, 8 weeks after induction. At that time, the expression of AQP1 significantly increases in the retina. In this study, we found a significant increase of AQP1 in the AH of patients with DM, which indicated that the function of the retina had changed at the stage of DM. The increase in AQP1 may reflect a change in the type of AQP4s expressed in glial cells. However, a previous study showed that AQP1 expression was lower in the AH of DM patients without DR than in controls. It is difficult to explain the reason for the difference between our findings and the previous ones, but it may have occurred because of racial differences or the different duration of DM, 2.36 years in our study compared with 7.7 years in the previous study. We speculate that the expression of AQP4 in the AH may not change in the early period of DM but increases significantly in the time DM before the occurrence of DR. We cannot know the timing of the change in AQP4 expression, which needs further study.

The effect of AQP4 on water metabolism in the retina is believed to rely on the formation of a protein complex with Kir4.1, which allows movement of water into and out of the cell. Kir4.1 is a K+ channel that is colocalized with AQP4 water channels in Müller cell membranes that surround the retinal vessels and at both limiting membranes. In animal models of DM, the expression of Kir4.1 protein around the vessels and at both limiting membranes is absent, similar to the location at which AQP4 is switched to AQP1. Some studies demonstrated that decreased Kir4.1 expression or function in the retina or vitreous body in DM may be associated with Müller cell activation and cause an imbalance in K+ concentration, leading to neuronal excitation, glutamate toxicity, and neuronal death. However, Midena et al. found that Kir4.1 levels in the AH were higher in patients with DM but may be a biomarker of ARC in patients without DM and a biomarker for retinal glial cell activation in patients with diabetes who do not have cataracts.
with diabetic macular edema than in healthy patients. In addition, the best improvement of corrected visual acuity after subthreshold micropulse laser treatment was associated with decreased levels of Kir4.1 and GFAP.6 The results are different from our study and previous studies. We cannot explain the reason and propose that more study of the mechanism of Kir4.1 in patients with DR is required. Increased GFAP expression in the retina is a marker for glial cell activation and an early sensor of retinal stress.27,28 We found significant increases of GFAP in the AH of patients with NPDR and PDR, which is consistent with previous reports.

Certain study limitations should be considered. First, this was a single-center study with a relatively small number of participants. Multicenter data or a larger sample size would be needed to further confirm our conclusions. Second, as hypertensive patients were excluded, we could not know the effect of hypertension on the level of the proteins we assayed in AH. The inclusion of hypertensive patients would help us to understand the relationship between hypertension and activation of glial cells. Third, two populations of macrogial cells in the retina, astrocytes and Müller cells, expressed AQP1, AQP4, Kir4.1, or GFAP, so the cellular origins of these proteins were unknown. Last, the study size did not allow stratifying the ARCs as nuclear, cortical, or posterior subcapsular cataracts. Consequently, any differences in AQP1 that occurred in the types of ARCs were not detected.

In summary, we found that the concentration of AQP1 in the AH was increased in patients with cataract and was also increased by DM and DR. We showed that AQP1 may be a biomarker of ARCs in patients without DM and a biomarker for retinal glial cell activation in diabetes patients without cataracts. The diverse expression of AQP1, AQP4, GFAP, and Kir4.1 in the AH at different stages of DR may help us to further understand the activation of glial cells in DM and DR.

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References

1. Grauslund J, Green A, Sjölak IE. Prevalence and 25-year incidence of proliferative retinopathy among Danish type 1 diabetic patients. Diabetesologia. 2009;52:1829–1835.
2. Liu L, Wu X, Liu L, et al. Prevalence of diabetic retinopathy in mainland China: a meta-analysis. PLoS One. 2012;7:e45264.
3. Pardue MT, Allen RS. Neuroprotective strategies for retinal disease. Prog Retin Eye Res. 2018;65:50–76.
4. Araujo RS, Santos DF, Silva GA. The role of the retinal pigment epithelium and Muller cells secretome in neovascular retinal pathologies. Biochimie. 2018;155:104–108.
5. Rungger-Brandl E, Dosso AA, Leuenberger PM. Glial reactivity, an early feature of diabetic retinopathy. Invest Ophthalmol Vis Sci. 2000;41:1971–1980.
6. Reichenbach A, Wurm A, Pannicke T, Iandiev I, Wiedemann P, Bringmann A. Muller cells as players in retinal degeneration and edema. Graefes Arch Clin Exp Ophthalmol. 2007;245:627–656.
7. Vujosevic S, Micera A, Bini S, Berton M, Esposito G, Midena E. Aqueous humor biomarkers of Muller cell activation in diabetic eyes. Invest Ophthalmol Vis Sci. 2015;56:3913–3918.
8. Midena E, Bini S, Martini F, et al. Changes of aqueous humor Muller cells’ biomarkers in human patients affected by diabetic macular edema after subthreshold micropulse laser treatment. Retina. 2020;40:126–134.
9. Tripathi RC, Millard CB, Tripathi BJ. Protein composition of human aqueous humor: SDS-PAGE analysis of surgical and post-mortem samples. Exp Eye Res. 1989;48:117–130.
10. Grus FH, Joachim SC, Pfeiffer N. Proteomics in ocular fluids. Proteomics Clin Appl. 2007;1:876–888.
11. Sohn HJ, Han DH, Kim IT, et al. Changes in aqueous concentrations of various cytokines after intravitreal triamcinolone versus bevacizumab for diabetic macular edema. Am J Ophthalmol. 2011;152:686–694.
12. Fischbarg J. Water channels and their roles in some ocular tissues. Mol Aspects Med. 2012;33:638–641.
13. Barandika O, Ezquerro-Inchausti M, Anasagasti A, et al. Increased aquaporin 1 and 5 membrane expression in the lens epithelium of cataract patients. Biochim Biophys Acta. 2016;1862:2015–2021.
14. Sharma S, Bollinger KE, Kodeboyina SK, et al. Proteomic alterations in aqueous humor from patients with primary open angle glaucoma. Invest Ophthalmol Vis Sci. 2018;59:2635–2643.
15. Qin Y, Xu G, Fan J, Witt RE, Da C. High-salt loading exacerbates increased retinal content of aquaporins AQP1 and AQP4 in rats with diabetic retinopathy. Exp Eye Res. 2009;89:741–747.
16. Qin Y, Ren H, Hoffman MR, Fan J, Zhang M, Xu G. Aquaporin changes during diabetic retinopathy in rats are accelerated by systemic hypertension and are linked to the renin-angiotensin system. Invest Ophthalmol Vis Sci. 2012;53:3047–3053.
17. Wilkinson CP, Ferris FL, III, Klein RE, et al. Proposed international clinical diabetic retinopathy and diabetic macular edema disease severity scales. Ophthimolology. 2009;110:1677–1682.
18. Chylack LT, Jr. Wolfe JK, Singer DM, et al. The Lens Opacities Classification System III. The Longitudinal Study of Cataract Study Group. Arch Ophthalmol. 1993;111:831–836.
19. Iandiev I, Pannicke T, Reichel MB, Wiedemann P, Reichenbach A, Bringmann A. Expression of aquaporin-1 immunoreactivity by photoreceptor cells in the mouse retina. Neurosci Lett. 2005;388:96–99.
20. Iandiev I, Pannicke T, Reichel A, Wiedemann P, Bringmann A. Diabetes alters the localization of glial aquaporins in rat retina. Neurosci Lett. 2007;421:132–136.
21. Fukuda M, Nakanishi Y, Fuse M, et al. Altered expression of aquaporins 1 and 4 coincides with neurodegenerative events in retinas of spontaneously diabetic Torii rats. Exp Eye Res. 2010;90:17–25.
22. Zhang Y, Xu G, Ling Q, Da C. Expression of aquaporin 4 and Kir4.1 in diabetic rat retina: treatment with minocycline. J Int Med Res. 2011;39:464–479.
23. Pannicke T, Iandiev I, Wurm A, et al. Diabetes alters osmotic swelling characteristics and membrane conductance of glial cells in rat retina. Diabetologia. 2006;55:633–639.
24. Bringmann A, Pannicke T, Uhlmann S, Kohen L, Wiedemann P, Reichenbach A. Membrane conductance of Muller glial cells in proliferative diabetic retinopathy. Can J Ophthalmol. 2002;37:221–227.
25. Olsen ML, Sontheimer H. Functional implications for Kir4.1 channels in glial biology: from K+ buffering to cell differentiation. *J Neurochem*. 2008;107:589–601.

26. Luo Q, Xiao Y, Alex A, Cummins TR, Bhatwadekar AD. The diurnal rhythm of insulin receptor substrate-1 (IRS-1) and Kir4.1 in diabetes: implications for a clock gene Bmal1. *Invest Ophthalmol Vis Sci*. 2019;60:1928–1936.

27. Picconi F, Parravano M, Sciarretta F, et al. Activation of retinal Muller cells in response to glucose variability. *Endocrine*. 2019;65:542–549.

28. Coughlin BA, Feenstra DJ, Mohr S. Muller cells and diabetic retinopathy. *Vision Res*. 2017;139:93–100.