Int J Clin Exp Pathol 2016;9(3):3322-3330
www.ijcep.com /ISSN:1936-2625/IJCEP0021357

Original Article

Ocular aquaporin-4 reverses up-regulation of glial fibrillary acidic protein in mice retina following elevated intraocular pressure

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Received December 7, 2015; Accepted February 17, 2016; Epub March 1, 2016; Published March 15, 2016

Abstract: Purpose: Changes in the expression of aquaporin-4 (AQP4) have been reported in various diseases. However, such changes and mechanisms remain unclear and need to be evaluated for the retinal injury. This study was performed to examine the expression changes in AQP4 and investigate the effect of AQP4 on activation of retinal glial cells following the elevated intraocular pressure (IOP). Materials and methods: The elevated IOP model was established by episcleral veins cauterization. Rebound tonometer was used to measure the IOP. Male wild-type moues (WT) and AQP4 knockdown moues (AQP4-/-) divided into different groups after episcleral veins cauterization according to the time of establishing models (1, 3, 7, 14 and 28 days). Hematoxylin-eosin (HE) staining was performed to evaluate the changes of retinal pathology. The expressions of AQP4 and glial fibrillary acid protein (GFAP) in WT and AQP4-/- moues following the elevated IOP were observed by immunofluorescence staining assay. Results: Our results found that the expression of AQP4 was significantly increased in WT moues following the elevation of IOP after 7, 14 and 28 days. HE staining showed that WT and AQP4-/- moues retina began thickening at 3 days after elevation of IOP, and the retinal ganglion cells were edema at 7 days after elevation of IOP. However, the retinal injury induced by elevation of IOP in AQP4-/- moues was significant attenuate which compared with that of WT moues. Since 1 day after elevation of IOP, the expression of GFAP was remarkably increased both in WT and AQP4-/- moues and the peak of WT moues was more obvious than that of AQP4-/- moues. Conclusions: The enhancement of AQP4 expression is associated with elevation in IOP and induces retinal injury by up-regulation of GFAP in retinal glial cells, suggesting that AQP4 may be a new target for treatment of glaucoma.

Keywords: Aquaporin-4, glial fibrillary acidic protein, glaucoma, retinal glial cells

Introduction

Fluid movement in brain is fundamentally important in normal brain and clinical relevant physiological and pathological conditions. Brain edema plays a role in the pathophysiology of central nervous system and produces elevated intracranial pressure, leading to brain ischemia and death [1]. The discovery of aquaporin water channels had led to the first molecular-level information about water transporting mechanisms in brain. The aquaporins are integral membrane proteins, widely present in the animal, plant and microorganism, involving in fluid transport in different cell types [2]. AQP4 is one of the most important subunits in aquaporins family and is strong expressed in the glial limiting membrane and astrocyte foot processes [3, 4]. AQP4 is also found highly expressed in cornea and lens epithelial cells, suggesting that AQP4 participates in production and regulation of aqueous humor, and visual and nerve transduction [5].

Retina is a part of central nervous system and consists of 90% of retinal glia cells, plays a critical role in the development and survival in neuron. Astrocytes and Müller glia are two retinal glia cells and make contact with retinal neurons, providing stability to the neural tissues [6], and induce blood-brain barrier properties within the vascular endothelial cells [7, 8]. Glial fibrillary acid protein (GFAP), a marker protein of astrocytes, functions as a monitoring index in activation of astrocytes [9]. Under the normal conditions, Müller glia has no obvious expres-

sion of GFAP. However, it is highly expressed in chronic elevated IOP rat model and in several pathophysiology of retina such as ischemic and hypoxic conditions [10-12].

Glaucoma is characterized by progressive loss of visual function result from death of retinal ganglion cells associated with elevated intraocular pressure (IOP). In the glaucomatous optic neuropathy, glia cells from the retina and optic nerve show abnormal behavior, resulting from the expression of GFAP in Müller glia and appearance of reactive astrocytes [13]. GFAP expresses in Müller glia and astrocytes in glaucomatous retina indicates that these cells may be involved in the apoptosis process, followed by increase in NO and TNF-α produced by glia cells, which lead to the death of retinal ganglion cells exposed to stressful conditions [10, 14].

Animal models of glaucoma aimed at reducing IOP and validating proposed gene targets for drug discovery are important in testing therapies. Several mouse strains have been identified spontaneously elevated in IOP [15]; however, they have some disadvantages compared to an induced glaucoma model. On the one hand, the elevated IOP is moderate and takes place slowly over half to one year [16]. On the other hand, IOP elevation presences in both eyes so that comparisons must be done between different mice, thus requiring more mice for study [17].

Overexpression of AQP4 is associated with ischemia and hypoxia in brain and induces injury. Strong expression of AQP4 in retina glia cells are key risk factors in the development of glaucoma. However, the expression of AQP4 upon retinal injury has not been fully understood. Furthermore, the hallmark of glaucoma is the death of retinal ganglion cells, especially retinal glia cells, leading to irreversible blindness. The present study analyzes a mice model of elevated IOP in the eye. The aim was to determine concurrent responses of retinal glia cells, using AQP4 and GFAP special antibodies as markers in retinal glia cells. Knockdown of AQP4 also used in this study to detect the association of AQP4 and GFAP in elevation of IOP-induced retinal injury.

Materials and methods

Regents

Monoclonal anti-AQP4 antibodies were from Millipore (Temecula, CA, USA), and secondary antibodies (goat anti-rabbit conjugated FITC) were from Invitrogen (Carlsbad, CA, USA). Phosphate buffer saline (PBS), albumin from bovine serum (BSA), chloral hydrate and DAPI were from ZSGB-Bio (Beijing, China).

Animals

Care of laboratory animals and animal experimentation were performed in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. All animal studies were approved by the Animal Ethics Committee of Nanjing Medical University. Experiments were performed on 10-14 weeks male moues (30-32 g) obtained from Nanjing Medical University of professor gang Hu (Nanjing, China). Two groups of moues were considered for study: wild-type (WT) moues and AQP4 knockdown (AQP4-/-) moues.

Induction of experimental glaucoma

Moues were deeply anesthetized with intraperitoneal injection of chloral hydrate (0.1 ml/10 g). The method was created according to the method of Ruiz-Ederra and Verkman [17]. Briefly, under an operating microscope, an incision was made through cutting the conjunctiva along the limbal periphery of the right cornea, followed by exposed and isolated the underlying episcleral veins. A caustic microforceps at high temperature was positioned under the episcleral veins until blockage was observed as marked venous engorgement without signs of leakage.

Intraocular pressure measurement

At 1, 3, 7, 14 and 28 days after surgery, IOP was measured with rebound tonometer under light general anesthesia. The measurement probe was perpendicular to the surface of cornea and 3 independent measurements were averaged to give a confidence interval of more than 99 percent.

Histology

At 1, 3, 7, 14 and 28 days after surgery, eyes following the elevation of IOP and control eyes were dipped into the 1% paraformaldehyde for 24 h. The cornea, iris and lens were removed under the operating microscope. For histological examined, 5 μm sections of fixed embedded tissues were cut and placed on glass slides, deparaffinized, and stained sequentially

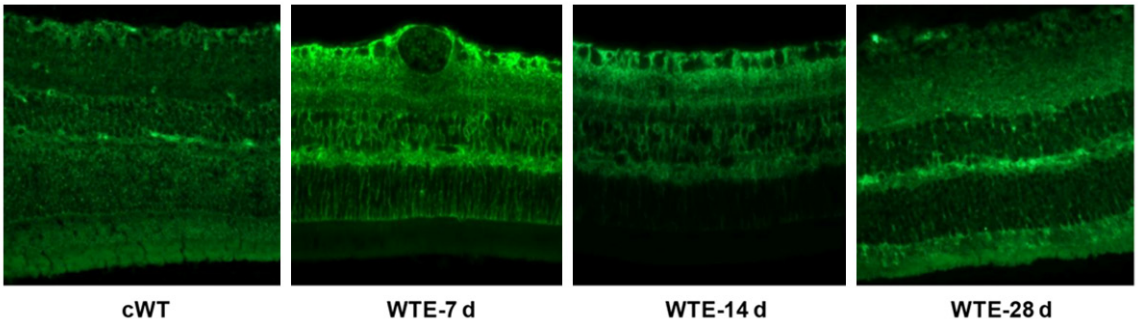

Figure 1. Expression level of AQP4 in WT moues with elevation of IOP. After induction of experimental glaucoma, AQP4 protein level in WT mouse retina with elevation of IOP was examined by immunofluorescence staining. 7, 14 and 28 d are the time periods of the sampling after elevation of IOP. cWT, WT moues without treatment; WTE, WT moues with elevation of IOP.

with HE. Stained tissue sections on slides were analyzed under identical light microscope.

Immunofluorescence staining for AQP4 and GFAP

Paraffin-embedded eye tissues sections were deparaffinized and hydrated for histological assessment. After antigen retrieval, the tissues were permeabilized with 0.1% Triton X-100 (Sigma-Aldrich, Shanghai, China) in PBS. After blocking with 1% BSA in PBS for 30 min, they were incubated with anti-AQP4 antibody, anti-GFAP antibody and corresponding FITC conjugated secondary antibody before staining the nuclei with DAPI.

Statistical analysis

Data for the statistical analysis were processed in a GraphPad Prism 5. All experiments were repeated at least three times with three replicates per condition each time. The paired, two-tailed Student's t-test was used to analyze the significance of difference between groups. Data were presented as means ± SD with the significant differences shown as $*P<0.05$.

Results

Elevated IOP increases the expression of AQP4 in moues

Several studies have been showed that AQP4 is highly expressed in the retinal glial cells especially in astrocytes and MIüller glial cells. Therefore, the effect of elevation of IOP on AQP4 expression in retina was tested. As shown in **Figure 1**, elevated IOP resulted in an increase in AQP4 protein after 7 days in ganglion cell layer (GCL), inner nuclear layer (INL), out nuclear layer (ONL), inner plexiform layer (IPL) and outer plexiform layer (OPL) as determined by immunofluorescence staining, which compared with the untreated control WT moues (cWT). These results suggest that elevated IOP induced retinal injury is an associated increase in expression of AQP4 in moues.

AQP4 Knockdown attenuated the elevated IOP in moues

Having documented significantly up-regulation of AQP4 in elevated IOP in rat retina, we wonder how AQP4 influences retinal injury induced by elevated IOP. We introduced the AQP4 knockdown moues (AQP4-/-) to investigate the biological functions associated with the retinal injury (**Figure 2A**). Firstly, we examined the IOP in two group moues, WT moues and AQP4-/- moues, respectively. As shown in **Figure 2B**, IOP significantly increased in WT moues eyes with episcleral veins burn compared to the control eyes and was maintained for up 28 days post-surgery. The experimental eyes showed a remarkably and sustained increase in the IOP during the experimental period. Three days before surgery, the average baseline IOP was 6.48±1.16 mm Hg and 6.55±0.90 mm Hg respectively for control and treated eyes, which had no significant differences. However, the average IOP in the treated eyes increased and peaked at 12.60±1.53 mm Hg.

AQP4-/- moues also resulted a remarkably and sustained increase in the IOP during the experimental period (**Figure 2B**). Three days before

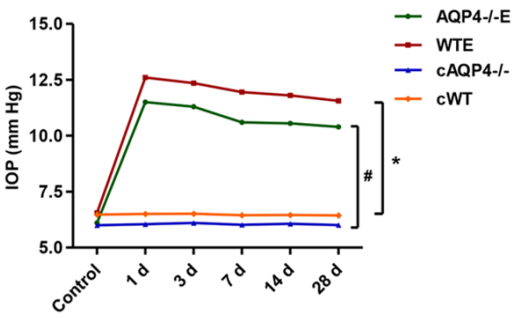

Figure 2. IOP in control and experimental glaucoma induced mice retina. cAQP4-/-, AQP4 knockdown moues without treatment; AQP4-/-E, AQP4 knockdown moues with elevation of IOP; cWT, WT moues without treatment; WTE, WT moues with elevation of IOP. *P<0.05 compared with cWT; #P<0.05 compared with cAQP4-/-.

surgery, the average baseline IOP for control and treated eyes was respectively 6.00±1.09 mm Hg and 6.10±1.11 mm Hg, which had no obvious significances. However, the average IOP in the treated eyes increased and peaked at 11.50±1.60 mm Hg. Interestingly, the increase in IOP of AQP4-/- moues was notably lower than that of WT moues, suggesting that AQP4 knockdown may attenuate the elevation of IOP in retinal moues with episcleral veins burn.

Histology

For both WT and AQP4-/- moues, HE staining was observed with ordered arrangement of the retina in control WT (cWT) and control AQP4-/- moues (cAQP4-/-). However, both WT and AQP4-/- moues showed retinal thickening 3 days after surgery, and became obviously at 7 days after surgery with retinal ganglion cell edema, cytoplasm became bright and empty, and nerve filer cell and inner nuclear cell thickening. After 14 days, the retina gradually shrinking and part of the ganglion cell nuclei was smaller and deeply stained (**Figure 3A** and **3B**). Interestingly, the retinal injury induced by elevation of IOP in AQP4 knockdown moues showed suppression compared with the WT moues with elevated IOP, indicating that AQP4 knockdown may attenuate the retinal injury induced by elevation of IOP.

AQP4 positive regulates GFAP in elevated IOP in moues

Whereas AQP4 levels appeared to increase in the retina with elevated IOP, based on the immunofluorescence results, it was possible to detect qualitative changes in immunoreactive GFAP levels in the retinal moues with elevated IOP. In the control group, immunostaining for GFAP was not visible in the WT and AQP4-/- moues. In the group with chronic elevated IOP, a great number of stained cells were visible in the WT and AQP4-/- moues as compared with those of the control group (**Figure 4A** and **4B**). However, the expression of GFAP in AQP4-/- moues with elevated IOP was significantly decreased compared with that in WT moues with elevated IOP. In addition, the pattern of AQP4 was similar to the pattern of GFAP in WT and AQP4-/- moues with elevated IOP, suggesting that AQP4 is positive regulation of GFAP following the activation of retinal glial cell and induces retinal injury through affecting glial microenvironment.

Discussion

Glaucoma is a major cause of blindness worldwide that results in progressive apoptosis in retinal ganglion cells. Activation of glia cells showed variety of morphology and structure, and associated with retinal ganglion cells and development and progression of diseases [18]. Since developing research in glaucoma, the animal models of glaucoma are more and more of considerable importance in therapies. Rats exposed to light condition moderated elevation of IOP and had no cytotoxicity of retina, which similar to the clinical patients with glaucoma. However, it had no significances because the elevation of IOP taken place slowly [19]. Scleral intravenous injection of hypertonic saline is another way to develop the model of glaucoma with a long maintenance for 200 days, but it is not widely used because of the small eyes in moues [20]. Under developing induction of mice models of glaucoma, we introduced a mice model of elevated IOP based on the episcleral vein cauterization method. After surgery, the IOP was significantly increased in WT moues compared with the control moues without episcleral vein cauterization, suggesting that episcleral vein cauterization is effective for develop a mice model of chronic elevated IOP.

AQP4 is one of the most important subunits in aquaporins family and is strong expressed in the Müller glia and astrocytes. It is characterized by insensitive to mercury and unregulated by antidiuretic hormone, functions in transportation of water [21]. In the decade years, the

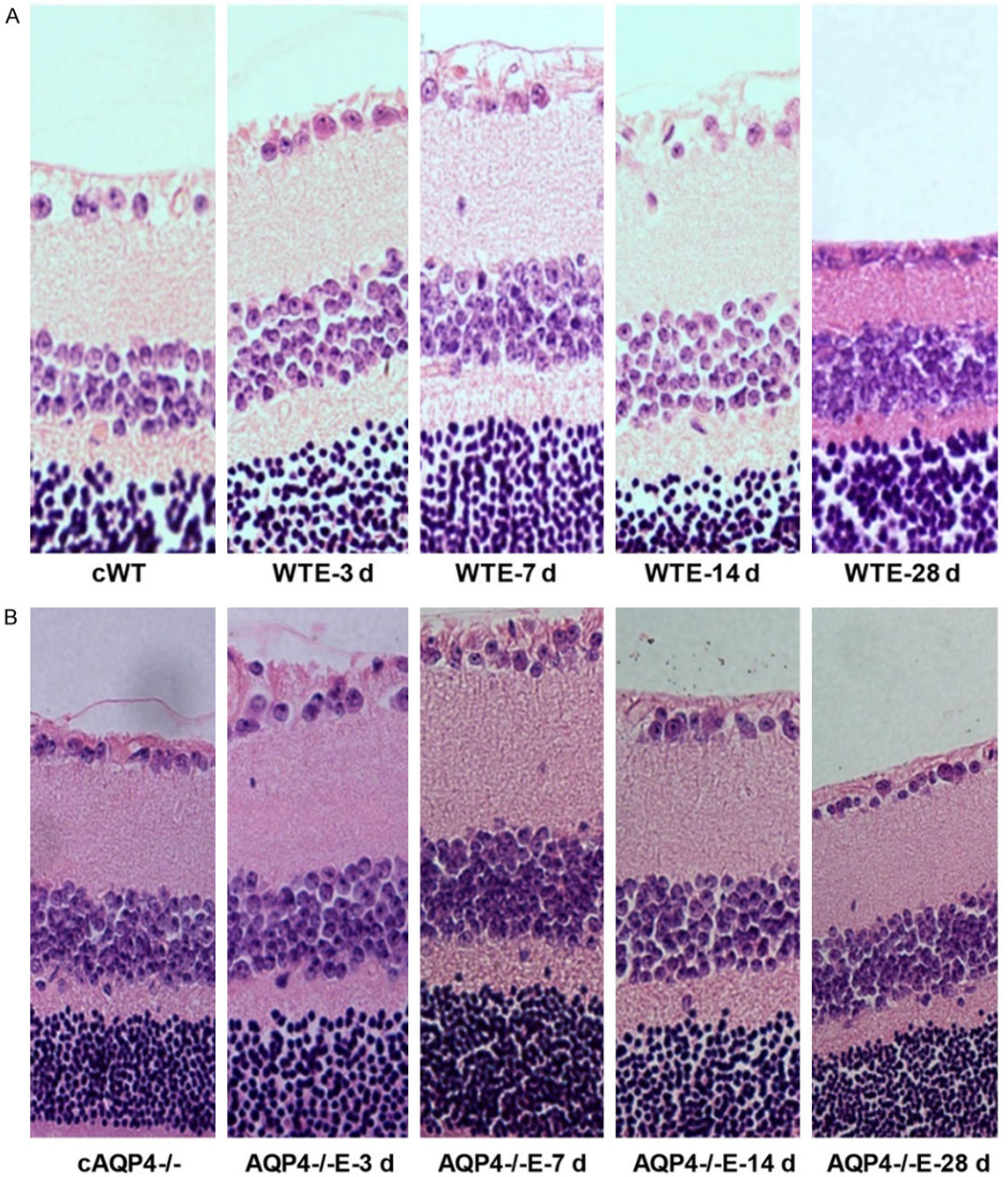

Figure 3. HE staining in control and experimental glaucoma induced mice retina. Histology of WT (A) and AQP4-/- (B) mice retina with elevated IOP. 3, 7, 14 and 28 d are the time periods of the sampling after elevation of IOP. cWT, WT moues without treatment; WTE, WT moues with elevation of IOP; cAQP4-/-, AQP4 knockdown moues without treatment; AQP4-/-E, AQP4 knockdown moues with elevation of IOP.

distribution and function of AQP4 in CNS and retina have been understood, however, the mechanisms of signaling transduction are still unknown. C-type natriuretic peptide (CNP) is considered to be a nervous mediator, which up-regulates AQP4 mRNA level in cultured astrocytes [22]. In the current study, elevation of IOP observed to up-regulate the expression of AQP4 level in the retina, suggesting that IOP elevation is an important factor for increasing the level of AQP4. Whether the up-regulation of AQP4 contributes to the development of glaucoma is not

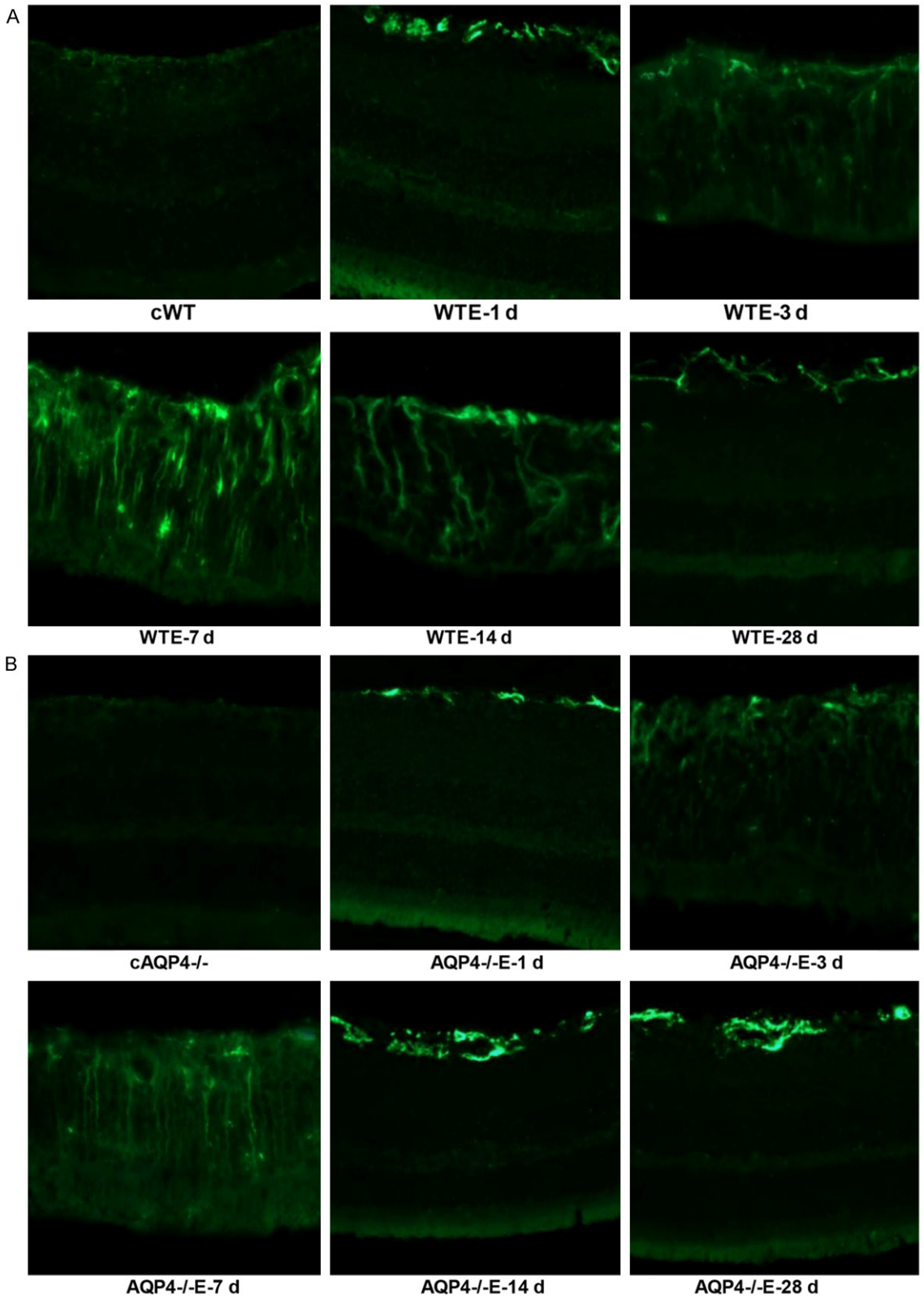

Figure 4. Expression levels of GFAP in control and experimental glaucoma induced mice retina. After induction of experimental glaucoma, GFAP protein levels in WT (A) and AQP4-/- (B) mice retina with elevation of IOP was examined by immunofluorescence staining. 1, 3, 7, 14 and 28 d are the time periods of the sampling after elevation of IOP. cWT, WT moues without treatment; WTE, WT moues with elevation of IOP; cAQP4-/-, AQP4 knockdown moues without treatment; AQP4-/-E, AQP4 knockdown moues with elevation of IOP.

clear and may require a selective AQP knockdown in retina. On the basis of those data, we introduced an AQP4 knockdown mice (AQP4-/-) to investigate the association with glaucoma.

The intraocular pressure in AQP4-/- moues was no significance compared with the WT moues after the episcleral vein cauterization. Although, there is contradictory report showing AQP4-/- moues has a lower of intraocular pressure compared with the WT moues [23]. However, the intraocular pressure was significantly increased in WT and AQP4-/- moues with episcleral vein cauterization, and the increased tension in AQP4-/- moues was notably attenuated than that in WT moues, suggesting that AQP4 knockdown may inhibit the elevation of IOP induced by episcleral vein cauterization.

In addition, AQP4 deletion is neuroprotective in retinal injury mice induced by transient ischemia [24] and is in astrocytes *in vitro* induced by hypoxia [25], suggesting that the reduction of AQP4 levels may be a beneficial mechanism. In agreement with these findings, our results showed that WT and AQP4-/- moues observed retinal thickening and retinal ganglion cell edema, cytoplasm became bright and empty, and nerve filer cell and inner nuclear cell thickening. However, these injuries resulted from elevation of IOP were markedly attenuated in AQP4-/- moues compared with WT moues. Similar findings have been reported in AQP null mice brain and are associated with reduced blood-brain barrier osmotic permeability, suggesting the possibility of AQP4 inhibitors reducing cytotoxic brain edema [26].

GFAP of astrocytes is known as an early marker for retinal injury and is widely used as an index of glial cell hypertrophy [27]. By immunofluorescence, we demonstrated an increase in GFAP protein levels in WT and AQP4-/- moues retina with elevated IOP. Increasing GFAP labeling was similarly observed at the rat optic nerve head exposed to elevated IOP [28]. Interestingly, Barber et al. reported that a differential GFAP expression pattern was found in the macroglial cells of retina in diabetes rats [29]. Numbers of vivo and vitro studies have shown GFAP plays an important role in several astrocyte functions inducing proliferation, differentiation, astrogliosis and inhibition of cerebral ischemia [30, 31]. However, the expression of GFAP was significantly higher in WT mouse retina compared with that in AQP4-/- mouse retina, suggesting that the activation of retinal glial cells can be indicated by GFAP and is associated with AQP4 expression. Moreover, there was a concomitant increase in the expression of GFAP and AQP4, equivalent in the expression of AQP4 and retinal injury induced by elevated IOP, suggesting that AQP4 contributes to the severity of retinal injury through regulating the retinal glial cells microenvironment.

Conclusion

In summary, the present study reported a finding of increased expression of AQP4 and GFAP in elevated IOP mice retina that may be link to glaucoma. AQP4-/- moues showed that retinal injury inhibition and expression of GFAP attenuation in elevated IOP mice retina, suggesting that AQP4 may act as a target for prevent and treatment for glaucoma.

Acknowledgments

This work was supported by the Scientific Project of Nanjing Medical University (2012-NJMU255).

Disclosure of conflict of interest

None.

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