

Apoptotic retinal ganglion cell loss is accompanied by complement and cytokine response in the β B1-CTGF primary open-angle glaucoma mouse model

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Glaucoma is a multifactorial disease and occurs in many different species. In humans, glaucoma is accounted one of the leading causes for blindness worldwide. Due to glaucoma's complexity, it is still unclear what pathomechanisms may be involved in its development in humans as well as in other species, such as canines. Diagnosis of glaucoma can be delayed because patients often do not notice a visual field loss until approximately 30% of retinal ganglion cells (RGCs) are lost (Kerrigan-Baumrind et al., 2000). Although the exact underlying pathomechanisms of glaucoma disease are not fully understood yet, an increased intraocular pressure (IOP) is related to RGC death and is considered the main risk factor. To understand the underlying mechanisms more precisely, appropriate animal models are needed. For glaucoma research, many ocular hypertension models are available. In most of them, elevated IOP is introduced through surgical interventions, like injection of microbeads, laser coagulation, or cauterization of episcleral veins (Dey et al., 2018). Further, the most used genetic ocular hypertension model, the DBA2/J mouse, reflects more the secondary pigment dispersion glaucoma form rather than primary open-angle glaucoma (POAG; John et al., 1998). Hence, an ocular hypertension glaucoma model, which mimics POAG and does not need a surgical induction is of great interest for researchers.

In the last years, many studies show that transforming growth factor (TGF- β 2) levels are elevated in the aqueous humor of glaucoma patients (Ozcan et al., 2004), hence, the involvement of TGF- β 2 in the trabecular meshwork outflow pathway moved more into the focus. It was noticed that the effects of TGF- β 2 on trabecular meshwork cells, such as increased contractility, formation of actin stress fibers, and the expression of α -smooth muscle actin, were mediated through the connective tissue growth factor (CTGF), which is a regulatory protein of the CCN family (an acronym for CTGF, cystine rich protein, and nephroblastoma overexpressed gene) (Junglas et al., 2009). Based on these findings, Junglas et al. (2009) established a new transgenic mouse model, where CTGF was specifically overexpressed in the lens. They noticed a wide opened chamber angle in 1 to 3 months old transgenic β B1-CTGF mice with intact ciliary body, iris, trabecular meshwork, and Schlemm's canal. Starting at an age of 1 month, β B1-CTGF mice developed a significantly increased IOP, which was constantly higher than in wild-type (WT) control mice. This was accompanied by a significant loss of axons in the β B1-CTGF optic nerves. They concluded that the overexpression of CTGF modulated the actin cytoskeleton of the trabecular meshwork causing POAG in the eyes of transgenic β B1-CTGF mice (Junglas et al., 2012). Due to these promising results, the effects of the CTGF overexpression on the retina were then well

characterized by several studies (Reinehr et al., 2019a, 2021; Weiss et al., 2021).

Elevated intraocular pressure and glaucomatous damage in β B1-CTGF mice: In accordance with findings by Junglas et al. (2009), Reinehr et al. (2019a) also reported an elevating IOP with increasing age of the β B1-CTGF mice. Here, the IOP of young β B1-CTGF mice at 5 and 10 weeks was comparable to WT mice, whereas after 15 weeks, the IOP was significantly higher in β B1-CTGF mice with a mean IOP of 17.49 ± 1.09 mmHg compared to WT (11.09 ± 0.20 mmHg; $P < 0.001$). Further, increasing apoptotic mechanisms were noted in young β B1-CTGF mice, resulting in a significant loss of RGCs in 15-week-old β B1-CTGF animals ($P = 0.02$; Figure 1A; Reinehr et al., 2019a). Even though RGC counts were not affected in 5- and 10-week-old β B1-CTGF mice, the apoptosis rate of these cells was already increased. Besides, a significant loss of neurofilament H was noted in 10-week-old transgenic mice. Also, as seen in RT-qPCR analysis, Casp3 mRNA levels (5 weeks: $P = 0.02$; 10 weeks: $P = 0.03$), as well as the Bax/Bcl-2 ratio mRNA levels were significantly increased in β B1-CTGF mice at 5 and 10 weeks. The depiction of apoptotic cells via TdT-mediated dUTP-biotin nick end labeling analysis on retinal cross-sections confirmed these findings (Weiss et al., 2021). The ongoing apoptotic mechanisms in young β B1-CTGF mice finally led to a degeneration of β B1-CTGF retinas in mice of an age of 15 weeks. Here, not only a significant loss of RGCs was found, but also a decline of cone bipolar cells. The retinal damage led to a disrupted retinal functionality via electroretinogram recordings. Thereby, the functionality of the photoreceptors (a-wave) can be separated from the cells of the inner nuclear layers (b-wave). This method revealed lower a- and b-wave amplitudes in 15-week-old transgenic mice, indicating a disrupted signal transmission in retinas of β B1-CTGF mice. These effects were not noted at 5 and 10 weeks of age. These findings were in accordance with the immunohistological and RT-qPCR analysis of cells of the inner layers (Reinehr et al., 2019a).

Interestingly, the synapses of β B1-CTGF mice were affected at a very early point in time. It was shown that the gephyrin⁺ area was significantly decreased in retinas of only 5-week-old β B1-CTGF mice ($P = 0.02$). Gephyrin colocalizes with GABAergic receptors and labels inhibitory synapses from RGCs to cells of the inner nuclear layer, such as bipolar and amacrine cells. However, the vesicular glutamate transporter transports glutamate into synaptic vesicles and is present in synapses between RGCs and cells of the inner nuclear layer. In β B1-CTGF mice, vesicular glutamate transporter was significantly lower at 5 ($P = 0.007$) and 10 weeks of age ($P = 0.01$; Weiss et al., 2021). For other neurodegenerative disorders, such as Alzheimer's disease, the loss of synapses is a

hallmark that leads to a compensatory mechanism to maintain synaptic connectivity. Since in the β B1-CTGF mouse model after 15 weeks the synapses were rather increased, a similar ongoing compensatory mechanism of the synapses might be possible. To assure these hypotheses further investigations need to be done.

Early activation of the complement system in β B1-CTGF mice: To further evaluate which mechanisms could lead to the cell loss in β B1-CTGF mice, the complement system and the cytokine response were examined. The complement system is part of the innate immune response and comprises about 50 proteins, which are activated through proteolytic cascades. It not only enhances the ability of antibodies and phagocytic cells to clear microbes and damaged cells, but it is also involved in promoting inflammation and is further associated with neurodegenerative diseases, such as multiple sclerosis and Alzheimer's disease (Gomez-Arboledas et al., 2021). The complement system can be activated via three different pathways. The classical pathway is activated when the complement component 1q (C1q) binds to an antibody of a foreign pathogen. The alternative pathway is initiated by the surface of pathogens itself, and the lectin pathway is activated when mannose-binding lectin recognizes specific sugars on the surface of pathogens. Many studies report the complement system to contribute to a diverse set of ocular diseases, such as glaucoma (Tezel et al., 2010). In glaucomatous human donor eyes, proteins that are associated with the complement system and to the different activation pathways, were found highly up-regulated (Tezel et al., 2010). In accordance with recent literature, an activation of the complement system was found in the retinas of β B1-CTGF mice. Interestingly, the complement activation was noted at an earlier point in time than the loss of RGCs, which occurred at 15 weeks of age. C3 ($P = 0.009$) and the membrane attack complex ($P = 0.03$), proteins of the terminal pathway of the complement cascade, were highly increased in β B1-CTGF mice at only 10 weeks of age. This indicates that not only apoptotic mechanisms, but also the complement cascade contributes to a subsequent loss of RGCs. The results of the complement system further show that not only one pathway is activated in β B1-CTGF mice but indicate an involvement of all three pathways at different points in time. It was found that the alternative pathway and the lectin pathway are both active, but at distinct stages. While factor B, as a representative of the alternative pathway, was upregulated in retinas of β B1-CTGF mice only at an age of 5 weeks, the mannose-binding protein-associated protein 2, which is part of the lectin pathway, was only increased in older β B1-CTGF mice at 15 weeks. Interestingly, the classical pathway, activated via C1q, was enhanced in β B1-CTGF mice at all investigated ages (5 weeks: $P = 0.003$; 10 weeks: $P = 0.04$; 15 weeks: $P = 0.03$) (Reinehr et al., 2021). Since the activation of the complement cascade occurred before RGC loss and simultaneously to the early ongoing apoptosis, it seems as if the complement cascade contributes to the retinal damage in the β B1-CTGF glaucoma mouse model.

In addition to all described effects on cells and the complement cascade, the cytokine response in the retina of β B1-CTGF mice was evaluated. An ongoing inflammation at a very early point in time was seen by an increased mRNA expression of interferon γ in β B1-CTGF mice of an age of 5 weeks ($P = 0.009$). In accordance with this, the mRNA expression of the C-X-C motif ligand (Cxl) 10, which is secreted as a response to interferon

γ, was increased at all ages (all $P < 0.05$). Further, an increased mRNA expression of Cxcl1 and Cxcl2 at 10 and 15 weeks indicated a consistent inflammation of the βB1-CTGF retinas.

Conclusion and future perspective:

Although glaucoma is one of the leading disorders responsible for blindness, the exact pathomechanisms still remain unclear. Currently, the modulation of the IOP is the only treatable factor, but this can only slow down, but not stop the degeneration. Therefore, new therapeutic approaches are urgently needed. To achieve this aim, suitable animal models which reflect the situation in human glaucoma as close as possible are crucial. For POAG, the βB1-CTGF mouse model seems to be a good candidate to study underlying pathomechanisms and explore novel diagnostic and therapeutic approaches. The overexpression of CTGF leads to a progredient increase of the IOP and an apoptotic RGC loss accompanied by a decrease of retinal function at 15 weeks of age. Before cell loss, an activation of the complement system and increased levels of pro-inflammatory cytokines could be observed (Figure 1B). These results point toward a crucial role of the immune response in the glaucomatous cell loss in the βB1-CTGF mouse.

Due to the gained results, we assume that the βB1-CTGF mouse can serve as a reliable model to study POAG. The slow progression of the IOP elevation in association with the RGC loss mirrors the findings in human glaucoma patients quite well. Hence, this seems to be a suitable model to investigate new treatments. One leverage point could be the inhibition of the immune system or parts of it, respectively. Previous studies had already shown that an inhibition or depletion of the complement system can be beneficial for the survival of RGCs (Reinehr et al., 2019b). For glaucoma patients, a systemic inhibition would be detrimental, hence, therapies should be delivered locally. In an IOP-independent model, the intravitreal application of a complement inhibitor against C5 already showed a protection of RGCs from glaucoma-like damage (Reinehr et al., 2019b). Further, other possible neuroprotective treatments using gene therapies might be of interest for glaucoma patients in the future.

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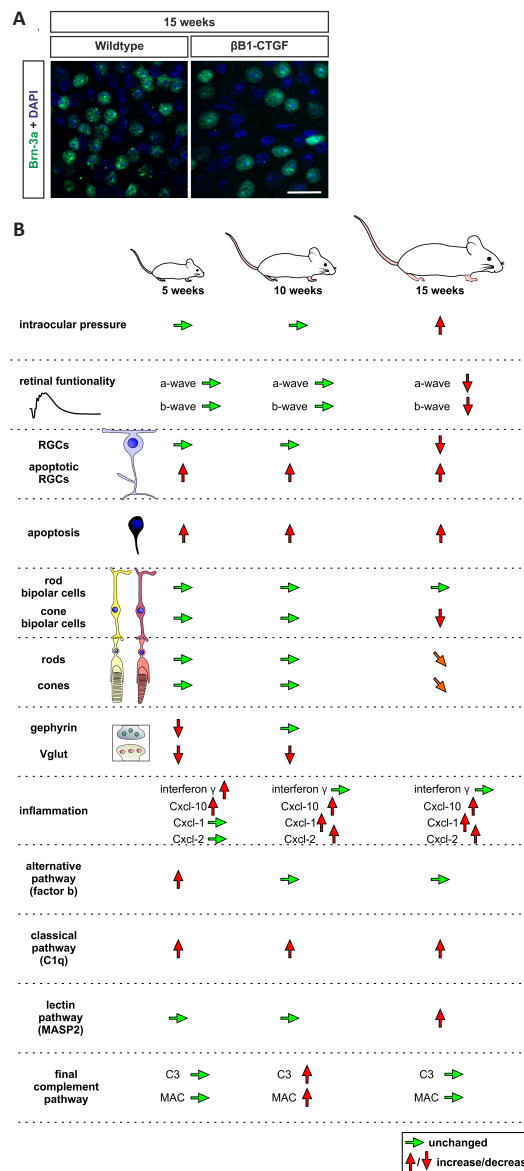


Figure 1 | Pathomechanisms occurring in the βB1-CTGF glaucoma model.

(A) Loss of retinal ganglion cells: in 15-week-old βB1-CTGF and corresponding WT controls, the number of RGCs was evaluated by staining flat mounts with an antibody against Brn-3a (green). DAPI (blue) counter-stained cell nuclei. Fewer RGCs could be noted in βB1-CTGF mice compared to WT controls. (B) Graphical summary of the pathomechanisms in the βB1-CTGF mouse model. At the age of 5 and 10 weeks, intraocular pressure, retinal function, and the number of RGCs were not altered in βB1-CTGF mice compared to WT controls. Nonetheless, at these ages, a higher apoptosis rate was observed. Interestingly, fewer synapses were observed, especially in younger transgenic mice. Further, activation of the complement system as well as an enhancement of pro-inflammatory cytokines, such as interferon γ or Cxcl-1 and 2, were observed in transgenic animals. At the age of 15 weeks, βB1-CTGF mice developed an increased intraocular pressure accompanied by a loss of RGCs and retinal function. In addition, cone bipolar cells and the mRNA expression levels of cones and rods were decreased at 15 weeks. Scale bar: 20 μm. Red arrows: Up-/down-regulation in βB1-CTGF mice compared to corresponding WT. C1q: Complement factor 1q; CTGF: connective tissue growth factor; Cxcl: C-X-C motif ligand; DAPI: 4',6-diamidino-2-phenylindole; MAC: membrane attack complex; MASP2: mannose-binding protein-associated protein 2; RGC: retinal ganglion cell; Vglut1: vesicular glutamate transporter 1; WT: wild-type. Green arrows: no alterations in βB1-CTGF mice compared to corresponding WT. Unpublished data.

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