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Immune Modulation in Glaucoma – Can Manipulation of Microglial Activation Help?

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1. Introduction

A large body of experimental results from clinical and experimental studies has strongly suggested an aberrant activity of the immune system in glaucoma. The roles of the innate immune responses in glaucoma are unclear and have been controversial about the concept of neuroprotection or neurodestruction. Protective immunity has been suggested occurring as a homeostatic response to injury, which can prevent disease progression. Neurodegeneration of retinal ganglion cells (RGCs) may be the consequence from a failure of proper controls for the initial immune response right after injury in some glaucoma patients. Long-term tissue stress in glaucomatous eyes appears to be important for the balance between neuroprotective and neurodestructive immunity. The onset, progression, and termination of retinal specific immune responses are predominantly regulated by the interaction among the RGCs and different glia (astrocytes, Müller cells and microglia) in the glaucomatous eyes. As immunocompetent cell in the central nervous system (CNS), microglia have diverse phenotypes and produce beneficial or destructive factors depending on the microenvironments they encounter. In response to injury, activated microglia have been shown to trigger neuronal death by producing high levels of cytotoxic factors such as nitric oxide, superoxide and tumor necrosis factor-α (TNF-α). However, increasing lines of evidence have shown that microglia can elicit protective effects by releasing trophic and anti-inflammatory factors. To transform microglia into neuroprotective or neurodestructive depends on the disease state or the type of stimulus to trigger them into "classically activated" proinflammatory (M1) or "alternatively activated" anti-inflammatory (M2) cells (Polazzi and Monti, 2010). *In vitro* study have shown that it is possible to manipulate the activation state of microglia so that their activation can be beneficial, i.e., protecting rather than destroying neurons (Polazzi et al., 2001; 2009). However, it is difficult to achieve this goal *in vivo*, especially in chronic neurodegenerative model. Our studies focus on modulating the retinal microglial cells by application of chemokine, monocyte-chemoattractive protein-1 (MCP-1/CCL2) into the posterior chamber to provide direct circumstance for attracting microglia to the retina. Furthermore, we also try to evaluate whether modulation of microglia in the retina can be achieved by the anti-aging Chinese medicine wolfberry. In this review, we will demonstrate how basic science research can be further developed and translated into pharmacological interventions through modulating the activation of microglia to prevent RGC loss in experimental glaucoma. This kind of approach can be one of therapeutic interventions for glaucoma patients in future.

2. Immunoregulation determines the fate of RGC in glaucoma

Glaucoma is an aging-associated neurodegenerative disease. In 2002, statistics gathered by WHO shows that glaucoma is the second leading cause of blindness worldwide, after cataract (Resnikoff et al., 2004). Glaucoma accounts for 12.3% of 37 million people affected by blindness, and 82% of which were 50 years or older. The number of glaucoma will increase to 79.6 million and the resulting blindness will increase to 11.1 million by 2020 (Quigley and Broman, 2006). Glaucoma results in irreversible loss of retinal ganglion cells (RGCs) and their axons, thus it is even a greater challenge than cataract for public health system. Therefore, investigation on potential neuroprotective agents is critical to prevent this blind leading visual impairment.

Glaucoma is defined as a group of optic neuropathies characterized by the irreversible loss of RGCs and their axons, accompanied by the excavation and degeneration of the optic nerve head (ONH) (Quigley, 1996). Elevated intraocular pressure (IOP)-related factors play an important role in initiation and progression of glaucoma. The glaucomatous neurodegeneration may be mediated via a combination of IOP-dependent compressive effects of the cribriform plates in the lamina cribosa on the axons of the RGCs, pressure-induced tissue ischemia, and various local cellular responses. The fate of RGCs may involve two or more cell stressors with additive or even synergistic effects. Increasing lines of evidence obtained from clinical and experimental studies strongly suggests diverse roles of the immune system in glaucoma as beneficial or destructive (Tezel, *2010,* doi:10.1016/j.exer.2010.07.009). It is now commonly recognized that there is an increased risks of failure in immune regulation under glaucomatous stress conditions (Schwartz and Ziv, 2008; Wax and Tezel, 2009).

2.1 Autoimmune neurodegeneration in glaucoma

Autoimmune neurodegeneration results from a failure to properly rectify an aberrant and stress-induced immune response. Neuronal antigens can initiate immune responses by recruiting cytotoxic T cells. This occurs primarily in low tension glaucoma patients as evident by abnormal T cell subsets (Yang et al., 2001a).

Humoral immune response is also involved in the onset and the progression of neurodegeneration in some glaucoma patients. There is an increased prevalence of monoclonal gammopathy (Wax et al., 1994), elevated serum titers of auto-antibodies to optic nerve head glycosaminoglycans (Tezel et al., 1999), auto-antibodies to retinal antigen such as rhodopsin (Romano et al., 1995), small heat shock protein (Tezel et al., 1998; Tezel et al., 2004), glutathione S-transferase (Yang et al., 2001b), gamma-enolase (Maruyama et al., 2000) and phosphatidylserine (Kremmer et al., 2001). Immunoglobulin has also been detected in the glaucomatous retina (Wax et al., 1998a). Heat shock protein antibodies (e.g. hsp60, hsp27, and α-crystallins) have direct pathological potential to facilitate apoptotic RGC death *in vitro* and *ex vivo* (Tezel et al., 1998; Tezel and Wax, 1999, 2000). In addition, clinical findings show that serum titers of auto-antibodies to heat shock proteins were independent of the severity of glaucomatous damage (Wax et al., 2001). Antibody-mediated neuronal damage may occur indirectly by molecular mimicry of self-antigen from pathogen (Romano et al., 1995; Wax et al., 1998b; Romano et al., 1999). This is supported by the finding of elevated auto-antibodies to bacterial heat shock proteins, including hsp60 (Wax et al., 1998b), and the increased expressions of HLA-DR/CD8 circulating T cells in low tension glaucoma patients (Yang et al., 2001a). In addition, significant alterations of serum Th1 and

Th2 cytokines are detected, suggesting abbreant immune responses of glaucomatous neuropathy (Huang et al., 2010).

2.2 Neuroprotective immune response – involvement of retinal microglia

Protective immunity has been suggested to occur as a homeostatic response to injury with the intent of preventing disease progression (Schwartz, 2007). The most important sites for immune modulation in glaucoma are retina and the optic nerve, which are thought to have an actively regulated immune privilege mechanism (Forrester, 2009). As the major immunocompetent cells in the CNS, the principal function of microglia is the quick response to the presence of pathogens and to CNS damage. In both human glaucomatous eye samples and animal models, the involvement of microglia in glaucoma has been reported (Schwartz et al., 2006). In human glaucomatous ocular specimens, microglia in the ONH and the parapapillary chorioretinal region of the ONH are activated and redistributed (Neufeld, 1999). In rat glaucoma model induced by cauterization, microglia in retinas exposed to chronic ocular hypertension appear as early as three days and last for about two months after IOP elevation (Wang et al., 2000; Naskar et al., 2002). Whether these retinal microglia play a protective or cytotoxic role in glaucoma deserve further investigation.

The balance between beneficial and deleterious immune responses is a critical issue for the treatment of neurodegenerative diseases. The use of immune-modulation drugs which are able to shift the immune response towards neuroprotection will be an effective therapeutic approach. As an FDA-approved drug for multiple sclerosis, glatiramer acetate (GA), also known as Copolymer-1 (Cop-1), was used as a treatment for autoimmune diseases and as a therapeutic vaccine for neurodegenerative diseases (Polazzi and Monti, 2010). GA is a synthetic oligopeptide of four naturally occurring amino acids, its activity derives from its ability to serve as a "universal antigen' that weakly activates a wide spectrum of self-reactive T-cells (Kipnis and Schwartz, 2002). T-cell-based vaccination with GA resulted in decreased plaque formation, reduction of excitotoxicity and induction of neurogenesis in Alzheimer's disease (AD) mouse model (Butovsky et al., 2006a; 2006b). This GA vaccination caused a phenotype switch in brain microglia to dendritic-like morphology, with the ability to produce insulin-like growth factor 1 (IGF-1) that counteracted the adverse Aβ-induced effects (Butovsky et al., 2006a; 2006b; 2007). Vaccination with GA significantly reduces loss of RGCs in rodent models of optic nerve crush injury, intraocular glutamate toxicity, glaucoma and macular degeneration (Schori et al., 2001). These results suggest that GA induced recruitment of dendritic-like microglia from bone marrow might contribute significantly to the anti-neurodegenerative effect. Vaccination has the impact on the entire body, how about using other immune-modulation drugs locally? Our laboratory has investigated the modulation of retinal microglia in a rat glaucoma model through intravitreous application of chemokine, monocyte chemoattractant protein-1 (MCP-1) (Chiu et al., 2010a). Furthermore, we also tested the involvement of retinal microglial cells in this glaucoma model which oral application of herbal medicine wolfberry polysaccharides have proved to be neuroprotective (Chan et al., 2007; Chiu et al., 2009). Our data showed that when the retinal microglia was tune to a neuroprotective state, named as police-state, there was a positive correlation with an increase in RGC survival under ocular hypertension (Chang et al., 2009).

3. Diverse activation of retinal microglia associated with divergent effects on retinal ganglion cell survival under chronic ocular hypertension (COH)

In the normal mature brain, microglia typically exist in a resting state that is highly ramified. In contrast to their non-moving cell body, processes of the "resting" microglia display high mobility, especially extension and retraction (Davalos et al., 2005). The brain microglia perform tissue surveillance and can patrol the entire neural parenchyma every few hours (Nimmerjahn et al., 2005). Use of Z-stack mode by multiphoton to scan different layers of retina from the nerve fiber layer to the outer segment on whole mounted retina allows us to observe the morphology of the resting state microglia. Similar to other reports in the brain, the resting microglia in the normal rat retina are highly ramified shape with small nuclei and long thin processes and was located in the inner retina with almost no overlapping of processes (diameter: ~50 μm, scattered throughout the retinal ganglion cell layer and the inner plexiform layer). They may also play a surveillance role in the retina (Chang et al., 2009; Chiu et al., 2009).

Upon activation, microglia emerged from a resting state and underwent morphological transformation from ramified to different activated forms, such as dendritic or amoeboid. Microglia constitute a family of cells with diverse phenotypes, some are beneficial and others are detrimental to surrounding cells (Schwartz et al., 2006). Like macrophages, microglia can exhibit different activated phenotypes: "classically activated" proinflammatory (M1) or "alternatively activated" anti-inflammatory (M2) (Benoit et al., 2008; Geissmann et al., 2008; Kigerl et al., 2009; Polazzi and Monti, 2010).

Although it has been shown that it is possible to manipulate the activation state of microglia *in vitro* so that their activation can be beneficial - protecting rather than destroying neurons (Polazzi et al., 2001; 2009). It is difficult to achieve this goal *in vivo*, especially in chronic neurodegenerative model. Our group managed to use a chronic ocular hypertension (COH) model to mimic glaucoma in rats. We tested differential activation of microglia in the retina under COH and their co-relationship with RGC survival in this chronic neurodegenerative model.

Adult female Sprague-Dawley (SD) rats (250-280 g) were grouped and used. Ocular hypertension (OH) was induced in the right eye of each animal using laser photocoagulation according to our previous publications (Chan et al., 2007; Chiu et al., 2007; Chiu et al., 2009; Chiu et al., 2010a; Chiu et al., 2010b). Schematic diagram (Fig. 1) shows the front and back view of laser photocoagulation applied (indicated as red spots) to the limbal veins (front veiw) and the three episcleral veins (two at the superior and one at the inferior quadrant, back view). After two weeks of laser photocoagulation, the limbal veins were replaced by scar tissue and could not be seen in the limbal area except the nasal 90 degrees where no laser photocoagulation was applied. The corneas of the OH eyes were healthy and transparent, with no neovascularization existed. The OH eyes were free of infection, cataract, intraocular bleeding or retinal detachment.

Photocoagulation using the argon laser increased the IOP of the right eyes (OH eyes) from the baseline around 15 mmHg to 22 mmHg for up to one month. There was significant loss of RGCs in the experimental eyes starting at 2 weeks after the first laser treatment. The density of RGCs was decreased from 2,241 to 1,964/mm^2 (#P = 0.002), this loss was only about 17% of total RGC in normal retina at one month after the first laser (Luo et al., 2009). Since ocular hypertension is not an acute injury causing massive neuronal death, microglia in the glaucomatous retina were not activated as they were in the optic nerve axotomy

model. The microglia detected in the inner retina of the OH eyes were in resting state under elevated IOP (Luo et al., 2009). The morphology of iba-1 positive microglia in the cross retinal sections was similar with the ramified one in the Naskar et al. (2002) study. The resting state morphology of microglia was also supported by Lam et al. (2003) in which they could not detect the phagocytic microglia in glaucomatous retina by using ED1 immunohistochemical staining. The microglial responses in this laser photocoagulation-induced COH model are similar to the situation in human glaucomatous retina where activated microglia are detected in the ONH and the parapapillary chorioretinal region of the ONH, where this region is not considered to be retina. Therefore, this model provides a good opportunity for us to investigate different morphologies of microglia modulated by different factors.

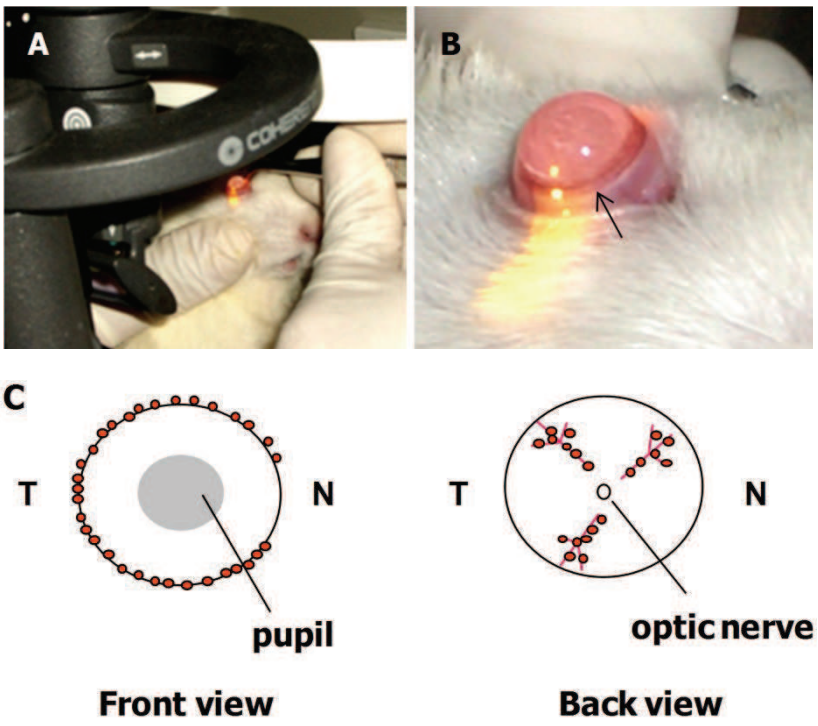

Fig. 1. **Photograph shows argon laser photocoagulation on the rat limbal and episcleral veins.** Right eye of the rat was photocoagulated by argon laser (A). Limbal veins of the eye (arrow) are indicated under slit-lamp (B). Schematic diagram (C) shows front view (observe from the corneal side) and back view (observe from the optic nerve side) of laser photocoagulation applied (indicated as red spots) to the limbal veins and three episcleral veins (two at the superior and one at the inferior quadrant). T: temporal; N: nasal.

3.1 Classical activation of retinal microglia is detrimental to RGC under COH

Conventional stimulation of microglia/macrophages by classical pathogens such as bacterial endotoxin lipopolysaccharide (LPS) or zymogen can be neurodestructive, because massive production of free-radicals triggered by these stimuli can induce both apoptosis and necrosis of neurons. Activated microglia have been considered to be endogenous malefactors in the CNS because they play important roles in neurological diseases such as Alzheimer's disease and amyotrophic lateral sclerosis (Sargsyan, et al., 2005). In response to injury, activated microglia have been shown to induce neuronal death by releasing excess cytotoxic factors such as superoxide (Lee, et al., 1993; Block, et al., 2007), nitric oxide (NO) and tumor necrosis factor-α (TNF-α) (Chang, et al., 2000a; 2000b; Colton and Gilbert, 1987). This kind of stimulation can eventually result in activation-induced cell death depleting the pool of these innate immune cells in the CNS. This is similar to the stimulation of LPS applied to the macrophages *in vitro*. LPS promotes the differentiation of "classically activated" M1 macrophages. These M1 macrophages secrete high levels of pro-inflammatory cytokines, release oxidative metabolites such as superoxide radicals (O^{2-}) and NO, and reduce production of neurotrophic factor (Benoit et al., 2008; Geissmann et al., 2008; Kigerl et al., 2009; Polazzi and Monti, 2010). Therefore, conventional stimulation of microglia/macrophages not only produces cytotoxic pro-inflammatory factors, but also depletes the pool of the innate immune cells to elicit possible neuroprotective effects.

In this COH model, a single intravitreous injection of 50 μg of LPS or MCP-1 1000 ng greatly alerted microglia to transform themselves from a resting state to a fully activated state in the glaucomatous eye up to four weeks under ocular hypertension (Chiu et al., 2010). The immunoreactivity of iba-1 in the microglia was dramatically increased. The microglia displayed enlarged nuclei and remarkably thick and short processes. With marked increase in the number of processes, their short processes displayed overlapping. Concomitantly, fully activated microglia were found in the nerve fiber layer and retinal ganglion cell layer corresponding to the area of retinal injury in animals with ocular hypertension. RGC loss was significantly increased from 17.4% in PB control OH retina to 28.3% in LPS group (# $p=0.007$). The increased RGC death should not be due to direct neurotoxic effect of LPS as it has been shown that LPS did not exert direct neurotoxicity (Bronstein et al., 1995). Dysregulated responses or over-activation of microglia is considered to be destructive and dangerous for neighboring neurons because of the harmful effects of free-radicals produced by fully activated microglia (Ladeby et al., 2005; Block et al., 2007).

3.2 Restricted activation of microglia is neuroprotective to RGC under COH

In contrast to conventional stimulation, activation of microglia/macrophages can be modulated by the cytokines secreted by infiltrated T-lymphocytes, or the local CNS environment, which is named as restricted (appropriate) stimulation. Immune suppressive cytokines that released from CD4+/CD25+ regulatory T cells or Th2 cells, such as IL-4, IL-10 or TGF-β can markedly modulate the activation state of microglia/macrophages (Kipnis et al., 2004). The macrophages are at an "alternative activated" M2 phenotype. These M2 macrophages promote angiogenesis, matrix remodeling and suppress destructive immunity (Sica et al., 2006).

MCP-1/CCL2 is a β-chemokine involved in the activation and recruitment of monocytic cells to injury sites (Zhang and Koninck, 2006). MCP-1/CCL2 can either induce neuroprotection or neurodestruction depending on the experimental model (Galasso et al., 2000; Eugenin et al., 2003; Kalehua et al., 2004). Using MCP-1/CCL2, the activation states of

microglia can be manipulated, they can exert divergent effects on RGC survival under COH. Our results demonstrated different morphologies of microglia, which are correlated to the severity of neuronal loss in an experimental rat glaucoma model. Compared to fully activated state of microglia in the retina (1000 ng MCP-1), a unique semi-activated phenotype of microglia was found in the 10/100 ng MCP-1 group(s) (Chiu et al., 2010). The nuclei were slightly enlarged and processes of microglia were significantly shortened with moderate thickening, and there was no overlapping of processes. Concomitant to the changes in microglial morphology, RGC loss appeared to be correlated with the activation status of microglia. Two weeks after the first laser, OH with intravitreous injection of PB resulted in 17.4% RGC loss. Injection of 10 ng of MCP-1 decreased the RGC loss to 10.3%, and 100 ng of MCP-1 significantly reduced RGC loss to 3.4% (** $p<0.001$, MCP-1 100 ng vs. PB). Further increase of MCP-1 to 1000 ng did not decrease but significantly increased RGC loss to 21.2%. At one month after the first laser treatment, compared to the PB control, MCP-1 100 ng significantly reduced RGC loss from 19.1% to 5.1% (* $p<0.001$).

The protective effects of microglia can be accomplished by producing neurotrophic molecules such as IGF-1 (Streit, 2005; Butovsky, et al., 2006a). Previous brain ischemia studies have shown that activated microglia can produce neurotrophic molecules such as IGF-1 (O'Donnell et al., 2002; Butovsky et al., 2006a; Lalancette-Hebert et al., 2007). Our study showed that after four weeks under COH, IGF-1 immunoreactivity was markedly reduced in the retinas with PB. In the 100 ng MCP-1-treated group, the immunoreactivity of IGF-1 was up-regulated and restored to normal level.

Similar phenomenon is also observed in oral feeding of herbal medicine, wolfberry polysaccharides, in this model (Chiu et al., 2009). One to 100 mg/kg LBP exerted the best neuroprotection and elicited moderately activated microglia in the inner retina with ramified appearance but thicker and focally enlarged processes. When activation of microglia was attenuated by intravitreous injection of macrophage/microglia inhibitory factor (MIF), protective effect of 10 mg/kg LBP was attenuated. Therefore, restricted activation of microglia under ocular hypertension is neuroprotective to the survival of RGCs.

4. Conclusion

Taken together, it is the time for us to reconsider how to categorize the activation state of microglia (Chang et al., 2009). First, the resting state with lots of processes and small size of cell body in morphology; second, the semi-activated state (police state or M2 phenotype) with thick and short processes and large size of cell body; third, the fully activated state (army state or M1 phenotype) with very thick processes and very large size of cell body with sometimes amoeboid shape. The army state of microglia can produce free-radicals including nitric oxide, superoxide and different pro-inflammatory cytokines. In contrast, the police state of microglia can produce trophic factor without releasing free-radicals. While resting microglia can be considered to be a security guard for immune surveillance, semi-activated (M2 activated) microglia can be regarded as a police officer to prevent any bad situation for further neuroinflammation and to protect citizen neurons without miss-firing by-stander neurons, and fully activated (M1 activated) microglia can function as army responsible to fight in a battle. Nevertheless, the by-stander neurons will be damaged unavoidably.

Our findings using the COH model, demonstrate a distinct morphology of microglia in response to neuroprotective dose of MCP-1/CCL2, and wolfberry. Definition of this distinct

morphology will help future studies to understand the biological mechanisms of neuroprotective microglia, opening up a new avenue of manipulating microglia to elicit neuroprotective effects in neurodegeneration.

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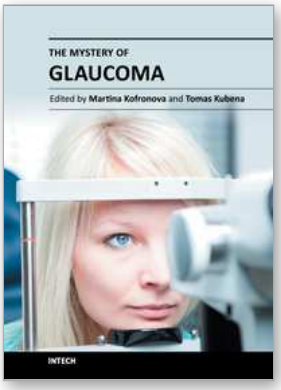

The Mystery of Glaucoma

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Since long ago scientists have been trying hard to show up the core of glaucoma. To its understanding we needed to penetrate gradually to its molecular level. The newest pieces of knowledge about the molecular biology of glaucoma are presented in the first section. The second section deals with the clinical problems of glaucoma. Ophthalmologists and other medical staff may find here more important understandings for doing their work. What would our investigation be for, if not owing to the people's benefit? The third section is full of new perspectives on glaucoma. After all, everybody believes and relies – more or less – on bits of hopes of a better future. Just let us engage in the mystery of glaucoma, to learn how to cure it even to prevent suffering from it. Each information in this book is an item of great importance as a precious stone behind which genuine, through and honest piece of work should be observed.

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