

Neuroprotective Agents in Glaucoma

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1. Introduction

1.1 Glaucoma as a neurodegenerative disorder

Glaucoma is a multifactorial disease in which multiple genetic, systemic and environmental factors interact to precipitate the disease. Increased intraocular pressure (IOP) is believed to be one of the major factors responsible for glaucomatous cell death. (Guo et al., 2005). The axons of the optic nerve make a twisting exit through the fenestrated collagenous barrier (the lamina cribrosa) before entering the brain. It has been proposed that increased IOP causes mechanical stress to the lamina cribrosa, which in turn exerts pressure on the axons that pass through it. This pressure may block the passage of essential material through the axons; which would lead to a slow degeneration of the axons of the retinal ganglion cells (RGCs) (Howell et al., 2007). With increased IOP, gradual withdrawal of trophic factor from RGCs has been observed in a mouse model. Also apoptosis was observed to be a significantly delayed in RGCs in such conditions (Johnson et al., 2000). It was therefore proposed that with increased IOP and mechanical stress on the lamina cribrosa, axons of RGCs are lost much earlier than the cell body of the RGCs. Thus RGCs become ineffective in their visual function much earlier than actual phenotypic changes are observed in the retina/optic disc, as they are incapable of sending visual signals to the visual center of the brain (Whitmore et al., 2005).

The primary goal of glaucoma treatment is to preserve vision. Elevated IOP as an important risk factor for glaucoma has continued to be a clinical focus for several reasons, including limited knowledge of the factors causing optic nerve damage, ease of measurement, the number of available IOP-reducing therapies, and the relationship of elevated IOP to disease progression. However, the relationship between IOP reduction and glaucoma damage is less clear, and such ambiguities suggest that factors other than IOP may be responsible for some of the long-term damage from glaucoma. Ocular blood flow in various tissues (e.g. retina, iris, optic nerve and choroid) was found to be reduced in glaucoma patients (Flammer et al., 2002). The blood flow reduction was more pronounced in Normal Tension Glaucoma (NTG) patients (Mozaffarieh et al., 2008). Interestingly, reduction of blood flow was also observed in the nail-bed capillaries of fingers in glaucoma patients; suggesting that global vascular dysregulation is involved in Primary Open Angle Glaucoma (POAG) especially in NTG cases.

Glaucoma is therefore no longer diagnosed by elevated IOP levels, and is now recognized as a neuropathy defined by characteristic optic disc and visual field change (Walland et al., 2006). IOP level is no longer relied on as a diagnostic criterion because 20% to 30% of glaucoma patients have IOP in the normal range (typically 10-21 mm Hg) (Sommer et al., 1991). Furthermore, there is annual progression even in patients treated with IOP-lowering

medical, laser, or surgical therapy. The Early Manifest Glaucoma Trial (Heijl et al., 2002; Leske et al., 2003) found that although the mean IOP during follow-up was significantly associated with the risk of progression, this risk was highly variable, and several other baseline factors were significant independent predictors whose combined effect might be as important as IOP. These additional independent predictors included presence of bilateral disease, worse mean deviation, degree of baseline exfoliation, age older than 68 years, presence of frequent disc hemorrhages, and duration between follow-up visits. The Ocular Hypertension Treatment Study found a relationship between IOP reduction and glaucoma incidence (Kass et al., 2002); however, progression was not confirmed in 85% of cases. (Greenfield & Bagga, 2005). The Collaborative Normal-Tension Glaucoma Study (1998a, 1998b) found that visual field progression could occur in both treated and untreated NTG patients, and no analyses of studies detected a relationship between a changes in the IOP and visual field progression. This clinical evidence of continued disease progression despite IOP management has provided the basis for proposed alternative risk factors and treatment approaches that could modify the clinical course of glaucoma.

Recent observations suggest that, in addition to RGCs, glaucoma patients have neurodegenerative lesions deep in the brain, supporting the hypothesis that it is a neurodegenerative disorder. In fact there are certain similarities between common neurodegenerative disorders and glaucoma (Ray & Mookherjee, 2009) which include:

- Loss of specific neuronal population. A common feature shared by neurodegenerative disorders is the loss of specific groups of neurons. For example, loss of cortical and hippocampal neurons in Alzheimer disease can be correlated with the loss of memory and cognitive function. After exiting from the eye, the axons of RGCs cross each other at the optic chiasm and terminate at the lateral geniculate nucleus (LGN). The LGN of each hemisphere represents the contralateral half of the visual field. Definite degenerative changes were observed in the optic tracts of the brains of glaucomatous patients and in the LGN. Also axonal loss was found in the intracranial optic tract and progressive degeneration in the visual cortex was observed along with the degeneration of RGCs (Gupta et al., 2006). Using Conventional MRI and magnetisation transfer imaging of the brain and optic pathway we also reached the same conclusion in our previous study (Kitsos et al., 2009). Thus, as in other neurodegenerative disorders a loss of specific groups of neurons involved in vision was observed in the glaucoma patients (Gupta et al., 2006, 2009; Yucel et al., 2001).
- Trans-synaptic spreading of neurodegeneration from RGC to cells deep in the brain. In neurodegenerative disorders, the disease spreads from sick neuron to healthy neurons through synaptic connections along functional and anatomical neural pathways, a process called *trans-synaptic* degeneration. Such degeneration is well known in Alzheimer disease and has also been found in human and experimental glaucoma cases (Gupta & Yucel, 2001). Studies have shown that RGCs damage in glaucoma is not confined to the primary insulted neurons, but that secondary injury follows which affects neighboring neurons as well. It is therefore believed that in glaucoma, treatment modalities that directly target both primary and secondary degeneration of the RGCs are required.
- Deposition of protein aggregates in glaucomatous RGCs as observed in other neurodegenerative disorders like Alzheimer's disease, Parkinson disease etc. Like other neurodegenerative disorders, deposition of unfolded or misfolded protein aggregates has been found in RGCs of glaucoma patients and there are indications that β -amyloid

- is involved in glaucoma pathogenesis (Gupta et al., 2008; Wang et al., 2008). Abnormal Tau protein, AT8 which is a hallmark of many neurodegenerative disorders has also been reported in glaucomatous RGCs (Gupta et al., 2008).
- Drugs administered in neurodegenerative disorders, treat glaucoma. Neuroprotective agents approved for the treatment of neurodegenerative diseases such as Alzheimer disease (Reisberg et al., 2003), are being assessed for the treatment of glaucoma to maximize recovery of injured RGCs and minimize secondary insults (Ettaihe et al., 1999).

1.2 The rationale of neuroprotection in Glaucoma

Neuroprotection refers to the use of any therapeutic modality that prevents, retards, or reverses neuronal cell death resulting from primary neuronal lesions. While neuroprotection refers to any therapy that prevents the death of existing RGCs after injury, neuroregeneration refers to any strategy that stimulates regrowth of an injured RGC axon. The idea of neuroprotection as a treatment strategy for glaucoma was borrowed from the neurological field, where the limitation of neuronal injury and protection from secondary insults spares neurons and improves clinical outcomes. This strategy has, however, not been successful in acute clinical neuronal injury, particularly with stroke. Nevertheless, for chronic neurodegenerative conditions, the strategy has made some small but important breakthroughs. Neuroprotection is similar to other cytoprotective therapies (eg, cardioprotection) in which the loss of the cell is targeted, not the disease process by which the loss occurs. For example, in cardioprotection, the cardiomyocyte itself is treated rather than the atheromatous plaque within a coronary artery that leads to myocardial infarction. Analogously, in glaucoma, an optic nerve disease, the RGC is treated rather than elevated IOP or other etiologies that indirectly cause the death of the RGC. Although IOP lowering and other such therapies can be considered indirectly neuroprotective, by strict definition and by comparison with other cytoprotective therapies, a neuroprotective therapy is directed at the neuron itself.

Glaucoma as all optic nerve diseases has an irreversible effect on vision because it causes death of RGCs and loss of their axons. As with all other neurons, once death of the RGC occurs, it is irreversible because mammalian neurons do not ordinarily replace themselves. Although the possibility of non-IOP-lowering therapy for glaucoma it was first recognized in 1972 (Becker et al., 1972) with the use of diphenylhydantoin for treatment of visual field loss in POAG, only recently have significant advances in the understanding of the mechanisms for death of retinal neurons occurred.

2. Potential neuroprotective strategies and agents in glaucoma

Several mechanisms, which are believed to initiate the apoptotic cascade in glaucoma and therefore are potential targets for neuroprotection include excitotoxicity, mitochondrial dysfunction, protein misfolding, oxidative stress, inflammation and neurotrophin deprivation (Baltmr et al., 2010). Therapeutic strategies targeting the mechanisms of cell damage listed above or the apoptotic cascade itself are reviewed.

2.1 Excitotoxicity

Most neurons (and also glia) contain high concentrations of glutamate (~10 mM) (Lipton & Rosenberg, 1994) after sequestration inside synaptic vesicles, glutamate is released for very

brief periods of time (milliseconds) to communicate with other neurons via synaptic endings. Once it is released in the pre-synaptic terminals it binds to a variety of receptor linked channels in the post-synaptic membrane, resulting in the influx of Ca^{2+} and the initiation of the action potential (Pin & Duvoisin, 1995). Glutamate is a major excitatory neurotransmitter in the central nervous system, including the retina (Thoreson & Witkovsky, 1999).

However, because glutamate is so powerful, its presence in excessive amounts or for excessive periods of time can literally excite cells to death. This phenomenon named glutamate receptor-mediated excitotoxicity has been associated with various diseases of the brain including Alzheimer's disease. Several studies have confirmed the neurotoxic effect of glutamate in the retina, while others have suggested glutamate-mediated RGC injury and death in glaucoma (Guo et al., 2006; Salt & Cordeiro, 2006). Increased buildup of glutamate was observed at the posterior aspect of the glaucomatous eye in monkeys with elevated IOP and in human glaucoma patients (Dreyer et al., 1996). Glutamate released from the apoptotic cell can trigger necrotic death of surrounding cells that have been spared from the original insult, initiating a cascade of autodestruction, further cellular injury and death (Casson, 2006; Cheung et al., 2008).

Once released from the pre-synaptic membrane, glutamate binds to specific postsynaptic receptors. There are three classes of glutamate-gated ion (or ionotropic) channels, known as (α -amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid AMPA), kainate, and N-methyl-D-aspartate (NMDA) receptors. Among these, the ion channels coupled to classical NMDA receptors are generally the most permeable to Ca^{2+} . Conventional NMDA receptors consist of two subunits (NR1 and NR2A-D), and more rarely NR3A or B subunits. There are binding sites for glutamate, the endogenous agonist, and glycine, which is required as a co-agonist for receptor activation (Johnson & Ascher, 1987). The receptor is probably composed of a tetramer of these subunits. The subunit composition determines the pharmacology and other parameters of the receptor-ion channel complex. Alternative splicing of some subunits, such as NR1, further contributes to the pharmacological properties of the receptor. Furthermore, the subunits are differentially expressed both regionally in the brain and temporally during development. Thus, developing antagonists selective for a particular subunit could be crucial in clinical practice.

Prolonged activation of glutamate receptors by increased levels of glutamate leads to high Ca^{++} level in the cytosol and thus facilitates the opening of mitochondrial permeability transition pore complex (PTPC) (Crompton et al., 1999). Opening of the PTPC will lead to apoptotic cell death (Kroemer et al., 2007). Increased activity of the enzyme nitric oxide synthase (NOS) is also associated with excitotoxic cell death. The neuronal isoform of the enzyme is physically tethered to the NMDA receptor and activated by Ca^{2+} influx receptor-associated ion channel, leading to increased nitric acid (NO) production. Increased levels of NO and the formation of toxic peroxynitrite ($ONOO^-$) have been detected in animal models of stroke and neurodegenerative diseases (Lipton et al., 1993). Importantly, elevations in extracellular glutamate are not necessary to invoke an excitotoxic mechanism. Excitotoxicity can come into play even with normal levels of glutamate, if NMDA receptor activity is increased, e.g., when neurons are injured and thus become depolarized (more positively charged); this condition relieves the normal blocking of the ion channel by Mg^{2+} and thus abnormally increases NMDA receptor activity (Zeevalk & Nicklas, 1992).

The role of energy expense in excitotoxicity has gained a lot of attention. With the disruption of energy metabolism during acute and chronic neurodegenerative disorders, glutamate is

not cleared properly and may even be inappropriately released. Elevated levels of glutamate subsequently results in increased concentrations of cytosolic Ca^{2+} and inorganic phosphate as well as decreased cellular ATP. (Crompton, 1999). This cellular state results in cell death; however, it has been observed that cells can remain viable in the presence of elevated Ca^{2+} , provided that ATP levels remain relatively unchanged (Broderick & Somlyo, 1987). Furthermore, extended elevation of cytosolic Ca^{2+} concentrations in the absence of exogenous adenine nucleotides can result in opening of the mitochondrial permeability pore (Crompton, 1999). Action potentials, consisting predominantly of the transport of Na^{+} by Na^{+} K ATPases, have been shown to consume approximately 50% of the total cellular energy used in the rabbit retina (Ames et al., 1992). As a result, energy compromised neurons cannot maintain ionic homeostasis and become depolarized.

Modulation of the NMDA receptor has been constituted a major area of research in glaucoma neuroprotection (Dong et al., 2008; Guo et al., 2006). In vivo and in vitro studies have suggested that blocking both the NMDA and the non-NMDA receptors simultaneously offers optimal protection against ischemic neurodegeneration (Mosinger et al., 1991). However, to be clinically acceptable, an anti-excitotoxic therapy must block excessive activation of the NMDA receptor while leaving normal function relatively intact to avoid side effects. Drugs that simply compete with glutamate or glycine at the agonist binding sites block normal function and therefore do not meet this requirement, and have thus failed in clinical trials to date because of side effects (drowsiness, hallucinations and even coma) (Lipton, 1993; Palmer, 2001).

MK801, is a non-competitive antagonist of the NMDA receptor and has demonstrated neuroprotective potential in the CNS for many years. MK-801 has also been found to protect RGCs both in vitro and in vivo in hypertensive rat models (Guo et al., 2006). Unfortunately, MK801 is not used clinically because of its neurotoxic effect, which are believed to be due to high affinity to the NMDA receptors and its long dwell time in the channel (Lipton, 1993).

Memantine also known as 1-amino-3,5-dimethyl-adamantane, is a moderate affinity, uncompetitive NMDA receptor antagonist. It is an 'open channel blocker', which means it can only bind within the NMDA ion channel if glutamate has previously bound to its receptor and induced the channel to open. Hence, when extracellular glutamate levels are elevated excessively, as is thought to occur in glaucoma, memantine becomes effective by having access to a site within the open NMDA (Osborne, 2009). Preliminary research into memantine as a neuroprotective agent in glaucoma demonstrated a reduction in NMDA-induced neuronal death in vitro and a protection of RGCs in several animal models of glaucoma (Lagrece et al., 1998), potentially via reduced cytochrome c release in the glaucomatous mouse retina (Ju et al., 2009). It appears that the compound enters the channel preferentially when it is (pathologically) activated for long periods of time, i.e., under conditions of excessive glutamate exposure (Chen & Lipton, 1997). Treatment with memantine also resulted in a reduction in the shrinkage of neurons within the contra-lateral LGB relay (layers 1, 4, and 6), a major target for RGCs (Yucel et al., 2006). So memantine had favorable activity in the channel to provide neuroprotection, while displaying minimal adverse effects, when administered to patients (Lipton, 1993). Memantine is Food and Drug Administration (FDA) approved for treating moderate to severe Alzheimer's disease (Reisberg et al., 2003) and is the only neuroprotective agent that has completed phase III clinical trial in patients with OAG. The trial showed that memantine was ineffective by the primary end point, with the variable mechanisms of retinal ganglion apoptosis being offered as an explanation (Osborne, 2008), although inadequate study design and inappropriate end point could be additional reasons for this result.

Bis(7)-tacrine a noncompetitive NMDA receptors antagonist had beneficial effect on glutamate-induced rat RGCs damage in vitro and in vivo (Fang et al., 2010)

Topiramate, a drug used clinically as an anti-epileptic was also shown to be protective against excitotoxic and ischemic retinal-neuron damage in vitro and in vivo (Yoneda et al., 2003).

Riluzole (2-amino-6-trifluoromethoxy benzothiazole) is a neuroprotective drug, which has been shown to block glutamatergic neurotransmission in the central nervous system (Martin et al., 1993), probably as a consequence of its action on ion channels. Riluzole could also prevent or decrease pressure-induced apoptosis and enhance ERG wave recovery in a pressure-induced ischemia animal model, highlighting the benefits of targeting multiple receptors in excitotoxic cell death (Ettaihe et al., 1999).

Galantamine, a small molecule acetylcholinesterase (AChE), which is used for the symptomatic treatment of Alzheimer's disease, protected RGCs in a rat glaucoma model. The neuroprotective effect of galantamine was superior to that conferred by memantine and occurred by activation of types M1 and M4 muscarinic acetylcholine receptors (Almasieh et al., 2010).

2.2 Mitochondrial dysfunction

Neurons, because of their high energy requirement, are heavily dependent on mitochondria for survival. Mitochondria not only constitute an energy-generating system, but are also critically involved in calcium signaling and apoptosis. Any malfunction of the mitochondrial electron transport chain results in an excessive generation of free radicals. When this overwhelms the intrinsic antioxidant capacity, amplified generation of free radicals results in the state of oxidative stress, which is evident in glaucomatous tissues. Mitochondria are morphologically dynamic organelles exhibiting a precise balance of ongoing fission and fusion during development and aging, which can be modified by disease (Santel & Frank, 2008). Mitochondrial fission is characterized by the conversion of tubular fused mitochondria into isolated small organelles, translocation of dynamin-related protein 1, and reduction of cellular ATP (Santel & Frank, 2008).

IOP elevation in vivo has been linked to mitochondrial damage in the optic nerve head by the promotion of mitochondrial fission, cristae depletion, and alterations in the expression and distribution of optic atrophy type 1 in DBA/2J mice (Ju et al., 2008). Intra-mitochondrial accumulation of Ca^{2+} , decrease in mitochondrial membrane potential and increase in membrane permeability have been implicated as a causative factor for RGCs apoptosis in glaucoma (Tatton et al., 2001; Tezel & Yang, 2004). Moreover, glaucoma-related stimuli such as hypoxia, TNF- α and oxidative stress can trigger the mitochondrial-mediated RGC death pathway.

Several compounds, have been proposed to enhance the available energy within the cell and prevent mitochondrial depolarization.

Nicotinamide is a non-toxic precursor of NADH, which is a substrate for complex I in the electron transport chain, a free radical scavenger and an inhibitor of poly-ADP-ribose polymerase (PARP). Dietary supplementation of nicotinamide in conjunction with creatine supplementation was shown to decrease the lesion volume induced by intracerebral injections of NMDA. PARP is a nuclear enzyme that is activated by breaks in the DNA chain and aids in minor DNA repair. The presence of excessive DNA damage initiates a PARP-mediated apoptotic response. The action of PARP results in the depletion of NAD and ATP, and ultimately cell death (Pieper et al., 1999). PARP inhibition, by 3-aminobenzamide, has

been shown to reduce neuronal damage in a high-pressure retinal ischaemia model. Of particular interest with regard to glaucoma, nicotinamide has recently been shown to attenuate ischaemic and phototoxic injuries to RGCs (Ji et al., 2008). It is important now to ascertain whether nicotinamide is equally beneficial in chronic models of RGC injury.

CoQ10, also known as Ubiquinone, plays an indispensable role in energy metabolism and offers the greatest potential as a neuroprotectant. It serves as a co-factor within the respiratory chain, carrying electrons and facilitating ATP production. It has been found to be highly effective as a neuroprotectant in animal models of neurodegenerative diseases such as Parkinson's disease (Beal, 2003). Its neuroprotective effect, demonstrated on RGCs both in vivo and in vitro, is believed to be multifactorial (Nakajima et al., 2008;) exerted not only through mediation of electron transport from complex I and II to complex III within the electron transport chain but also through its antioxidant properties, regulation of gene expression, and inhibition of PTP (Cheung et al., 2008).

There is increasing evidence in a variety of models that creatine may provide neuroprotection through metabolic energy buffering (Beal, 2003). Creatine is a guanidine compound that is found in meat and is also endogenously produced. Neuronal tissue has a very high ATP requirement for the generation of action potentials through the axonal ATP-dependent Na⁺/K⁺ ion transport system as stated previously. In order to replenish ATP levels, the phosphoryl-group from phosphocreatine is donated to ADP via the action of mitochondrial and cytosolic creatine kinase to produce ATP and creatine (Wallimann & Hemmer, 1994). There are two main isoforms of creatine kinase: cytosolic creatine kinase that balances energy distribution in the cytosol, and mitochondrial creatine kinase (Mi-Ck) that buffers energy between the mitochondria and the cytosol. (Brdiczka & Wallimann, 1994) There are several possible explanations for the neuroprotective qualities of creatine. First, creatine supplementation, which results in increased levels of phosphocreatine, may cause greater energy buffering during cell repair processes that occur during injury. Second, creatine may reduce cerebral ischemia infarct volume by improving cerebral blood flow (Prass et al., 2007). Third, the administration of creatine could result in a decreased release of glutamate. This may be achieved either through increased glutamate uptake into synaptic vesicles, which is an energy-dependent process, (Xu et al., 1996) or by an increased conversion of glutamate to glutamine (Bender et al., 2005). Finally, creatine supplementation may inhibit the opening of the PTP through the action of Mi-Ck (Beal, 2003; Kroemer et al., 2007). Creatine supplementation has provided significant neuroprotection against in vivo models of traumatic brain injury (Scheff and Dhillon, 2004), cerebral ischaemia (Lensman et al., 2006), Huntington's and Parkinson's disease. Concerning the neuroprotective effects of creatine against retinal/optic nerve neurodegenerative disorders, intravitreal injection of creatine partially rescues RGCs from NMDA-induced excitotoxicity (Schober et al., 2008).

2.3 Protein misfolding treatments

Amyloid deposits, consisting of aggregates of Ab, are a characteristic feature of several neurodegenerative diseases such as Alzheimer's, Parkinson's disease and mild cognitive impairment. They have also been recently implicated in the pathogenesis of retinal damage (Shimazawa et al., 2008), age-related macular degeneration (AMD) (Johnson et al., 2002) and glaucoma (Gupta et al., 2008; Wang et al., 2008; Guo et al., 2007).

Drugs designated to target b-Amyloid (Ab) include b-secretase inhibitors (BSI) such as N-benzyloxycarbonyl-Val-Leu-leucinal (Z-VLL-CHO) which has been found to reduce RGC apoptosis in vitro and in vivo, as well as Congo red and Anti-Ab antibodies (Guo et al., 2007)

). Triple therapy, targeting different stages of the Ab pathway using BSI, Anti-Ab antibodies and Congo red, showed a superior neuroprotective effect on RGC apoptosis in a rat ocular hypertension model compared to mono-therapy (Guo et al., 2007).

Also of interest with regards to protein misfolding are Heat shock proteins (HSPs), a group of specialized molecular chaperones which are upregulated in stressful conditions and mediate various physiological functions inside cells such as restoration of normal structural integrity (Soti et al., 2005). Several families of HSP have been implicated in neurodegenerative diseases, and glaucomatous RGC apoptosis (Guo et al., 2007), with increased levels of circulating autoantibodies to alpha-crystallins and HSP27 (Tezel et al., 1998), and increased immunostaining of HSP-60, HSP-27 in RGCs and the retinal blood vessels in glaucoma patients (Tezel et al., 2000). Considerable evidence also supports the involvement of HSPs, including HSP27, in intrinsic protection mechanisms of retinal cells in glaucoma. HSP27 is upregulated in experimental models of glaucoma, as well as in human glaucoma and the phosphorylation state of HSP27 is a critical determinant of its ability to act in a protective capacity as detected in glial cells. However, the exact biological mechanism is still unclear. HSP72 is an anti-apoptotic chaperone protein that may interfere with multiple stages of the apoptotic pathway. (Mosser et al., 2000). The preinduction of HSP72 in RGCs enhances the survival of RGCs under hypoxic, excitotoxic, and glaucomatous stress (Park et al., 2001). Systemic administration of Geranylgeranylacetone, an agent that has been used clinically for the treatment of gastric ulcers and gastritis, in the rat glaucoma model has been shown to increase the expression of HSP-72 with a marked reduction in RGC loss (Ishii et al., 2003).

2.4 Oxidative stress

Oxidative stress is a pathological condition in which the rate of reactive oxygen species (ROS) production exceeds the body's antioxidative capacity. Any malfunction of the mitochondrial electron transport chain results in an excessive generation of free radicals. When this overwhelms the intrinsic antioxidant capacity, amplified generation of free radicals results in the state of oxidative stress, which is evident in glaucomatous tissues.

Ischemia, potentially caused by vascular dysregulation and reperfusion injury to cells are critical inducers of oxidative stress (Flammer et al., 1999), leading to further ROS generation with ATP depletion and mitochondrial failure, triggering the caspase-dependent and caspase-independent mitochondrial cell death pathways. The increased levels of ROS enhance lipid peroxidation, protein peroxidation and single strand breaks in nucleic acids (Finkel & Holbrook, 2000). It has been proposed that after an initial insult to RGC axons at the optic nerve head, besides neurotrophin insufficiency, increased superoxide generation may also signal apoptosis of the RGC soma. Evidently, there is an increase in mitochondrial superoxide production within RGCs after axonal injury that is further amplified by oxidative stress (Nguyen et al., 2003). It is also evident that a shift to a reduced intracellular redox state induced by the use of sulfhydryl-reducing agents markedly prolongs RGC survival in *in vitro* and *in vivo* models of axonal injury (Swanson et al., 2005). RGC mitochondria regulate superoxide production differently from other neuronal cells, most likely as a result of differential expression and function of the mitochondrial electron transport chain components.

There is an increasing body of clinical as well as experimental evidence that oxidative stress contributes to the pathology of glaucoma (Izzotti et al., 2006; Tezel, 2006). Increased levels of free radicals have been measured in the retina and optic nerve head of animals with

experimental glaucoma. Free radical scavengers have been shown to protect against RGC death in various models of injury, while clinically glaucoma patients have been shown to have reduced antioxidant defenses (Izzotti et al., 2006). ROS have also been found to induce Muller cell activation and dysfunction, generating further oxidative material (Neufeld & Liu, 2003;). Furthermore, many retinal proteins exhibit oxidative modifications in experimental glaucoma, which may lead to important structural and functional alterations (Tezel et al., 2005).

Melatonin, a potent naturally occurring antioxidant with free radical scavenging activity, displays a critical role in aqueous humour circulation and demonstrated a neuroprotective effect on RGCs in vivo (Siu et al., 2004; Tang et al., 2006) via multiple mechanisms.

Derived from leaves of the *G. biloba* tree, Ginkgo biloba extract (GBE) has been used in traditional Chinese medicine for thousands of years, and has been one of the most prescribed drugs in Europe (Mahadevan & Park, 2008). It has been effectively used to treat diseases such as peripheral vascular disease (Mahadevan & Park, 2008), Alzheimer's disease (Yancheva et al., 2009), AMD (Rhone & Basu, 2008) and low-tension glaucoma (Quaranta et al., 2003). Ginkgo biloba extract (EGb761) contains two major compounds: 24% flavones glycoside and 6% terpenoids. The exact mechanism of the neuroprotective effect of EGb 761 is still unknown. However EGb 761 was found to be an excellent antioxidant, effectively inhibiting chemically induced apoptosis (Thiagarajan et al., 2002), as well as possessing both anti-inflammatory and antiplatelet activating factor activities. EGb 761 also enhances the cerebral blood flow and increases the ocular blood flow velocity (Chung et al., 1999). In a model of chronic glaucoma, GBE was shown to be neuroprotective for the pre-treatment and early post-treatment phases of glaucoma (Hirooka et al., 2004) and the few studies that have evaluated whether GBE may improve visual function, have produced promising results (Quaranta et al., 2003; Cheung et al., 2002). However, a National Health Interview study showed that there was no significant association between glaucoma patients and Ginkgo biloba use (Khoury et al., 2009).

2.5 Anti-inflammatory and immunological strategies

Growing evidence in clinical and experimental studies over the past decade strongly suggests the involvement of the immune system in glaucoma. Serum and tissue findings, including chronic activation of resident immunoregulatory glial cells, altered T-cell repertoires, increased autoantibody production, retinal immunoglobulin deposition, and complement activation, support the hypothesis that both innate and adaptive immune activity accompany glaucomatous neurodegeneration (Tezel & Wax, 2007).

Regarding T-cell cytotoxicity to RGCs, there is in vitro evidence that activated T cells may be directly cytotoxic to RGCs and induce RGC apoptosis mainly through death receptor-mediated signaling. Recent in vivo studies also support the feasibility of eliciting a T-cell-mediated experimental autoimmune model of glaucomatous neurodegeneration. In rats immunized with HSPs, RGCs progressively die, exhibiting a pattern with similarities to human glaucoma, including topographic specificity of cell loss. (Wax et al., 2008)

It is evident that the glial activation response in glaucomatous eyes involves activation of glial immunoregulatory functions and antigen-presenting ability. The expression of MHC class II molecules on glial cells, required for antigen presentation to T cells, is upregulated in glaucomatous eyes. Not only microglial cells, but also astrocytes exhibit HLA-DR immunolabeling in the glaucomatous human retina and optic nerve head (Tezel et al., 2003; Yang et al., 2001). Up-regulation of MHC class II molecules on rat glial cells and stimulation

of T cell activation in cultured retinal and optic nerve tissue have been demonstrated (Tezel et al., 2007). Glial MHC class II molecules are also upregulated in experimental animal models of glaucoma which may have neurosupportive and neurodestructive consequences. Increased expression of HSPs28 and ROS-dependent controlling pathways seem to be critical for the initiation of an activated immune response (Tezel et al., 2007, 2005). In addition to T-cell-mediated injury, autoantibody-mediated retinal damage has been associated with the pathogenesis of retinal diseases (Khan et al., 2006). Increased serum autoantibodies to various retina and optic nerve proteins have also been identified in patients with glaucoma over the past decade (Grus et al., 2008; Tezel & Wax, 2007) and retinal immunoglobulin deposition was seen in glaucomatous human donor eyes. There is evidence supporting the hypothesis that exogenously applied antibodies, including HSP antibodies, which exhibit increased serum titers in many patients with glaucoma, can be internalized by retinal neurons. At concentrations similar to those found in the patient serum, these antibodies can facilitate neuronal apoptosis (Tezel and Wax, 2000b). However, despite many immunologic associations, there is presently no direct evidence to confirm that neurodegenerative injury in glaucoma occurs as the direct result of aberrant cellular or humoral immunity.

Growing evidence implicates the involvement of the complement cascade in the neurodegenerative injury in glaucoma. Recent histopathologic studies of human tissues as well as in vivo studies using animal models have demonstrated that various complement components are synthesized during glaucomatous neurodegeneration (Stasi et al., 2006; Kuehn et al., 2006). In addition, terminal complement complex has been shown to be formed in the retina in both human and rat glaucoma (Kuehn et al., 2006).

Ongoing studies are testing an increasing number of innovative immunomodulatory strategies as a promising alternative to classic immunosuppressive treatments (Smith & Rosenbaum, 2000). One of the most efficient and specific ways to treat organ-specific autoimmune diseases has been based on systemic administration of native autoantigen or its altered peptide variants. Such strategies have been considered to be an important therapeutic option for the treatment of autoimmune diseases, because they selectively aim to deplete disease-inducing T cells in blood circulation or to trap these cells in peripheral lymphoid organs, thereby preventing them from invading the target organ. Antigen-based immunomodulatory treatments aiming to elicit immunogenic tolerance to tissue-specific antigens may also induce regulatory T cells. It has also been suggested that the loss of immunity to certain self-antigens or its insufficiency in the presence of increased levels of risk factors play an important role in neurodegenerative processes. For that reason, it has been proposed that vaccination could be a means of inducing the immune system to help eliminate many adverse factors associated with glaucomatous neurodegeneration and perhaps also supporting cell renewal and repair. Such a vaccine is thought to induce a beneficial immune response that recruits immune effector cells to counteract or neutralize some destructive factors, thereby preventing disease progression, although not its onset. Augmentation of immune system by passive transfer of T cells directed against myelin basic proteins or active immunization with the myelin-derived peptide reduces RGC loss after optic nerve injury (Schwartz, 2001).

Minocycline, a neuroprotective tetracycline derivative that suppresses chronic neuroinflammation and microglial activation, was shown to protect RGCs after optic nerve transection and in optic neuritis models (Maier et al., 2007). Furthermore, long-term and systemic treatment of DBA/2J mice with minocycline, commencing before clinical evidence

of glaucoma, suppressed retinal microglial activation and improved ganglion cell integrity (Bosco et al., 2008).

Copolymer-1 (Cop-1), also known as glatiramer acetate is a promising drug. It has been approved by the FDA to treat the Multiple sclerosis (MS). Cop-1 is a low affinity synthetic non-encephalitogenic analogue to myelin basic protein, triggering a neuroprotective autoimmune response, by binding to MHC proteins and cross reacting with various T cell and CNS myelin. Cop-1 displayed neuroprotective activity on RGCs in vivo in the rat model of optic nerve crush (Kipnis et al., 2000), in animal models of high IOP (Bakalash et al., 2003) and against glutamate-induced excitotoxicity (Schori et al., 2001). The neuroprotective effect in the rat model of glaucoma is believed to be mediated by increasing the number of T-Lymphocytes.

T-cell/glia/macrophage interactions should be well-controlled and synchronized and precise control mechanisms of the immune activity should be identified, so that these strategies will be clinical successful. For example, Monocyte chemoattractant protein-1 (MCP-1)/CCL2 (chemokine involved in the activation and recruitment of monocytic cells) was able to protect RGCs in experimental glaucoma. However, the appropriate dose of the drug was crucial for the neuroprotective effect (Chiu et al., 2010).

Targeting specific immunomediators involved in immunogenic injury constitutes another strategy for immunomodulation. It has been proposed that pro-inflammatory cytokines like TNF- α , which is produced in the brain and in the eye by microglial cells, play an important role in glaucomatous neurodegeneration (Tezel et al., 2001). Increased expression of TNF- α and its receptor were observed in the optic nerve heads of glaucoma patients. TNF- α , a potent proinflammatory cytokine, induces the production of NO which can be cytotoxic to the RGC (Tezel & Wax, 2000a). TNF- α activities are mediated via interaction with two distinct receptors, the death domain-containing TNF-receptor 1 (TNF-R1) which mediate the majority of TNF- α biological activity, and the non-death domain-containing TNF-receptor 2 (TNF-R2). The release of TNF- α and its subsequent binding to TNF-R1, triggers a caspase-dependant and a caspase-independent component of mitochondrial death-promoting pathways (Tezel et al., 2004). TNFR1 was shown to be involved in the neurodegenerative process of glaucoma (Tezel et al., 2001), neuronal cell loss and retinal ischemia, whereas TNF-R2 showed neuroprotective activity, reducing retinal ischemia (Fontaine et al., 2002).

Anti-inflammatory drugs, which target the TNF- α signaling pathway and display neuroprotective activity, have become an area of increasingly active investigation. Blocking TNF- α with anti-TNF- α neutralizing antibody or deleting the genes encoding TNF- α or its receptor prevented the effects of ocular hypertension in a mouse model (Nakazawa et al., 2006a). Opioid-receptor activation using morphine could reduce retinal ischemic/reperfusion-injury in vivo via suppression of TNF- α production (Husain et al., 2011). Finally, an antioxidant enzyme, peroxiredoxin 6 has shown to reduce TNF- α -induced and glutamate-induced RGC death in a rat model by reducing levels of reactive oxygen species, NF- κ B activation and intracellular calcium influx (Tulsawani et al., 2010).

2.6 Neurotrophin deprivation

Neurotrophic factors, small molecular weight peptides, which are widely expressed in RGCs, have an indispensable role in growth, differentiation and survival. They include: nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), Neurotrophins 3, 4 and 5 (NT3, NT4 and NT5), which exert their effects through tropomyosin related kinases (Trk). Several research studies demonstrated that the flow of neurotrophic factors from the

superior colliculus in the CNS to the RGCs is markedly reduced in the animal model of glaucoma, where both retrograde and anti-retrograde axonal transport are compromised (Hayreh et al., 1979; Rudzinski et al., 2004). This leads to a reduction in neuronal trophic support, which in turn compromises neuronal survival and triggers apoptosis, as seen in RGCs following transection of the optic nerve (Berkelaar et al., 1994).

There is substantial evidence that exogenous neurotrophic factors have a powerful survival effect on injured RGCs. Several groups have investigated the effect of intraocular injection of BDNF in models of RGC death. Intraocular delivery of BDNF protein (Ko et al., 2001) or gene transfer using adeno-associated virus (AAV) (Martin et al., 2003) led to RGC neuroprotection in experimental glaucoma induced by chronic intraocular pressure elevation. However, the effect of exogenous BDNF is temporary: it delays, but does not prevent, the onset of RGC death. (Di Polo et al., 1998; Mansour-Robaey et al., 1994). Administration of BDNF by repeated intravitreal injections or osmotic minipumps failed to extend the time-course of RGC neuroprotection (Mansour-Robaey et al., 1994). Delivery of BDNF by adenovirus-infected Muller cells provided a sustained source of NT, but led to only transient rescue of RGCs (Di Polo et al., 1998) which could be explained by the fact that TrkB mRNA and protein levels are markedly down-regulated in experimental glaucoma. These results suggest that reduced TrkB expression in injured RGCs contributes to their desensitization to exogenous, and possibly endogenous, BDNF. Based on this, a neuroprotective strategy was developed involving up-regulation of endogenous TrkB in RGCs by AAV-mediated gene transfer (Cheng et al., 2002). This study demonstrated a marked increase in the duration and level of BDNF-induced neuroprotection of axotomized RGCs upon up-regulation of TrkB. Specifically, TrkB gene transfer into RGCs, combined with exogenous BDNF administration, increased survival by 76% at 2 weeks after axotomy, a time frame in which 90% of these neurons are lost without treatment. This strategy substantially extended RGC survival compared with the shorter effect of BDNF protein alone (Di Polo et al., 1998; Mansour-Robaey et al., 1994). Recently, it has also been shown that the co-injection of an antagonist to leucine-rich repeat protein, LINGO-1, a known negative regulator of neuronal survival, with BDNF had a greater neuroprotective effect on RGCs than with BDNF alone (Fu et al., 2008a). Agonistic TrkB mAb causing sustained TrkB activation also delayed RGC death, and protected the retinal structure in optic nerve axotomy and in glaucoma animal models (Bai et al., 2010).

In addition to BDNF, other neurotrophic factors have shown efficacy in models of RGC injury. For example, exogenous ciliary neurotrophic factor (CNTF) protein enhanced RGC survival during elevated intraocular pressure (Ji et al., 2004). Intraocular injection of glial cell line-derived neurotrophic factor (GDNF) or neurturin was neuroprotective for axotomized RGCs, albeit with less efficacy than BDNF (Koeberle & Ball, 2002; Yan et al., 1999). Combined treatment of BDNF and GDNF resulted in increased RGC survival compared to independent administration of each neurotrophic factor (Koeberle & Ball, 2002; Yan et al., 1999). Interestingly, adenovirus-mediated CNTF gene transfer was reported to increase retinal CNTF and CNTF receptor levels, which correlated with increased survival of axotomized RGCs (van Adel et al., 2005). A novel approach to deliver CNTF *in vivo* involves the use of encapsulated cell intraocular implants. Cells transfected with the human CNTF gene are sequestered within capsules that can then be surgically implanted into the vitreous chamber of the eye. A semipermeable membrane in the implant allows CNTF to diffuse out and nutrients to diffuse in, while preventing immune cells from destroying CNTF-producing cells. A phase I safety clinical trial of this technology in patients with retinal degeneration was recently completed

without apparent ocular or systemic complications (Sieving et al., 2006). Thus, the use of encapsulated cell implants is a novel approach for retinal delivery of neurotrophic factors that may have future applications for glaucomatous and other optic neuropathies as well as other retinal diseases. Similarly, GDNF gene transfer using adenovirus or electroporation conferred protection of RGCs after optic nerve transection. (Ishikawa et al., 2005; Straten et al., 2002). More recently, intraocular injection of slow-release poly(DL-lactide-co-glycolide) microspheres containing GDNF was shown to promote RGC survival in an animal model of inherited glaucoma. (Ward et al., 2007).

Finally in a recent study it was shown that administration of eyedrops containing NGF was able to prevent RGC loss in an animal glaucoma model and furthermore to improve visual function in patients with advanced glaucoma. Although only three patients were included, the results are promising (Lambiase et al., 2009).

2.7 Apoptosis cascade

The structure and function of mitochondria are critical determinants of neuronal health, whereas mitochondrial dysfunction leads to RGC death through caspase-dependent and caspase-independent pathways, initiated by the loss of mitochondrial membrane potential, release of cell death mediators, and oxidative stress.

Mitochondria-related neuroprotective strategies were focused on the control of mitochondrial function by targeting the Bcl2 family. A caveat in the therapeutic targeting of mitochondria-mediated events is the reversal of the early steps of the cell death cascade. Most importantly, once the mitochondrial lipid bilayer is compromised after the mitochondrial translocation of Bax, cell death is inevitable, because already triggered events, the disruption of oxidative phosphorylation, loss in ATP production, increased generation of ROS, and release of cytochrome c constitute the crucial step that represents the point of no return for apoptotic cell death.

A promising agent for glaucoma therapy is BIRC-4, also known as XIAP (IAP: inhibitor of apoptosis protein). Intravitreal injection of adeno-associated viral vector using chicken-b-actin (AAV-CBA) coding for human BIRC4 in the rat model of chronic glaucoma resulted in marked reduction in RGC apoptosis that was sustained for 12 weeks. This neuroprotective effect is believed to be mediated either via direct inhibition of caspase-3 and caspase-8, indirectly by maintaining the neurotrophin production from Muller cells and influencing aqueous humour circulation or a combination of both (McKinnon et al., 2002).

Synthetic IQACRG peptide was delivered *in vivo* in order to work as an enzymatically inactive caspase mimetic which binds to caspase substrates as a pseudoenzyme and protects them from proteolysis by caspases. IQACRG significantly reduced NMDA-induced RGC death in culture and *in vivo* by inhibiting NMDA-triggered MMP-9 activity and preventing cleavage of MEF2C protein that would otherwise have been engendered by caspase activation preceding RGC death. (Seki et al., 2010)

2.8 Compounds with multiple/novel mechanisms of action

Brimonidine tartrate, is a third generation α_2 adrenergic agonist with weak α_1 activity. Animal studies demonstrated the presence of α_2 receptors in the retina or optic nerve head, laying the foundation for the potential neuroprotective role of brimonidine (Wheeler et al., 2001). Experimental models suggest that brimonidine confers neuroprotection in several types of ocular injury, including ischemia-induced injury, (Aktas et al., 2007; Danylkova et al., 2007) optic nerve compression or optic nerve crush injury (Levkovitch-Verbin et al.,

2000), photoreceptor degeneration, (Wen et al., 1996) and ocular hypertension and glaucoma (Hernandez et al., 2008; Wheeler et al., 2001). The mode of action of Brimonidine however remains unclear with various proposed mechanisms. The positive effect of Brimonidine on RGC survival, that includes a reduction in their soma size in a rat model of ocular hypertension, is believed to be mediated through the attenuation of glutamate toxicity by inhibition of NMDA receptor function (Dong et al., 2008) and/or the upregulation of brain-derived neurotrophic factors (Hernandez et al., 2008). However, in a rat model of pressure-induced retinal ischemia, it was suggested that Brimonidine's neuroprotective effect is mediated via the inhibition of the apoptotic cascade, possibly through the induction of anti-apoptotic genes such as bcl-2 and bcl-x, as well as extracellular-signal regulated kinases (ERKs) and phosphatidylinositol-3 kinase/ protein kinase Akt pathways (Lai et al., 2002). While Brimonidine's effect on RGCs in isolated rat retinas, as well as in vivo in rat and rabbit glaucoma models was also shown to be mediated through the reduction of α_2 -adrenoceptor mediated reduction of intracellular cAMP (Dong et al., 2008). A clinical trial assessing the non-IOP-related effects of Brimonidine, demonstrated reduced visual field deterioration in comparison to 360° laser trabeculoplasty (Gandolfi et al., 2003). The results of Brimonidine treatment in Low-Pressure Glaucoma Treatment Study are pending (Krupin et al., 2005).

Estrogens, cholesterol derived steroid hormones, maintain the normal function of various organs, with estrogen receptors ERa and ERb widely expressed in human and animal retinal tissues (Kobayashi et al., 1998). Estrogen has demonstrated neuroprotective effects in animal models of Alzheimer's and other neurological diseases (Hoffman et al., 2006). The neuroprotective effect of estrogen is believed to be mediated mainly via estrogen receptors ERa (Dubal et al., 2001;) and includes multiple mechanisms of action. 17 β -estradiol treatment of rat cortical neurons exposed to glutamate demonstrated increased neuronal integrity and function, mediated possibly by a reduction in the levels of caspase-3 and calpain (Sribnick et al., 2004). An estradiol analogue also demonstrated protective on the RGCs in vitro and in vivo (Nakazawa et al., 2006b;)

Endogenous erythropoietin/erythropoietin receptor (EPO/EPOR) system was shown to participate in intrinsic recovery mechanisms after retina injury. ERO, in addition to being a hematopoietic cytokine, demonstrated neuroprotective effect in various models of neurodegeneration. Furthermore, exogenous erythropoietin could rescue RGCs in an experimental ocular hypertension model (Fu et al., 2008b).

Candesartan an angiotensin II type 1 receptor (AT1-R) blocker showed significant neuroprotection against RGC loss in a rat chronic glaucoma model. (Yang et al., 2009) According to the same study, upregulation of AT1-R was associated with chronic elevated IOP. Consistent with that, the long-term use of angiotensin-converting enzyme (ACE) inhibitors, which are widely used as antihypertensive drugs, are believed to have a favorable effect on the visual fields in patients with normal-tension glaucoma (Hirooka et al., 2006) and angiotensin II receptor gene polymorphisms have been found in humans, and may be associated with the risk for glaucoma (Hashizume et al., 2005).

3. Challenges, limitations, clinical perspectives of neuroprotection

The treatment of glaucoma is no longer restricted to managing vision loss related to elevated IOP. The focus has shifted to reducing disease progression and loss of visual function that result from neurodegeneration. Alternative strategies that include neuroprotectants may be

useful in preventing optic nerve damage, thereby improving the structural, functional, and other patient-reported outcomes.

There is however substantial evidence that the results of preclinical neuroprotection studies fail to correlate with their clinical counterparts. Although numerous neuroprotective agents have been shown to limit neuronal damage in animal models of disease these encouraging preclinical results almost invariably fail to translate into clinical success. This lack of correlation between preclinical and clinical results could be due to various reasons (Danesh-Meyer & Levin, 2009).

A. Issues concerning the preclinical studies

- Lack of perfect animal model of glaucoma. Although animal models are useful tools for elucidating pathogenic mechanisms and testing the neuroprotective ability of new treatments, it is very likely that the initiating insults and pathogenic pathways vary between different experimental models and even more compared to humans. Because of this, animal models may not mirror human disease accurately since they often lack the heterogeneity inherent in human pathologic conditions. When an animal model for glaucoma is first designed, the goal is to produce a homogeneous reproducible optic nerve injury meaning loss of RGCs. However, the ideal model should produce focal injury to RGC axons at the optic nerve head, death of groups of RGCs in sectors in the retina with no loss of other retinal neurons. In clinical trials there is considerable heterogeneity, compounded by comorbidities, risk factors, polypharmacy, and minimal if any control over physiologic parameters. These variables may influence the efficacy of the potentially neuroprotective agent under investigation. These limitations are further exemplified by the contrast between the slow and multifactorial progressive onset of glaucoma in humans and its rapid or subacute induction in young healthy laboratory animals by pharmacologic agents or one particular intervention (for example obliteration of episcleral vessels). Furthermore, factors associated with glaucoma in humans, such as chronicity of injurious conditions and age-dependent dysregulation of tissue response mechanisms, may play a role in the cumulative deterioration of the homeostatic balance, thereby promoting the spread of neuronal damage, rather than favoring retained cell survival. As new biological pathways participating in glaucoma damage are discovered (for example activation of the glia), a critical question that arises, is which experimental model(s) accurately mimic various conditions in human glaucoma and are therefore useful for testing neuroprotective treatments, and for generating sufficient and compelling preclinical evidence in order to justify testing new agents in well-designed clinical trials.
- Dose. The appropriate dose of a potential neuroprotective drug for use in human subjects is difficult to extrapolate from animal studies due to differences in CNS microcirculation and structure, receptor and postreceptor signaling. The presence of the blood barrier in the nervous system makes bioavailability prediction more difficult. Furthermore, many neuroprotective drugs exhibit U-shaped curves in which concentrations higher or lower than optimum are toxic or ineffective. Lack of a complete dose-response curve, insufficient data on CNS penetration, inadequate assessment of therapeutic index, or a combination of these in preclinical studies could be responsible for clinical studies failure.
- Timing. Differences between preclinical and neuroprotection clinical trials relate also to the timing of the intervention. In animal models, the neuroprotective agent is

introduced relatively soon after induction of the disease, when neuroprotection may have its greatest effects, in contrast to human clinical trials, in which the patient is enrolled after glaucoma is well established and irreversible RGC loss is manifested by visual field defects. It is suggested that a window of time exists between the initial phases of injury and the final phases of cell death and only during this time frame, treatment with a neuroprotective agent could rescue injured neurons. Hence, the treatment drug may reach the neural tissue only after death of the neurons is already inevitable and because of this may be ineffective.

- **Study Design.** The methodology of preclinical study design and the statistical analysis of the results also differ significantly from clinical studies. For example, although randomization and masking are standard practices in phase III clinical trials, these techniques are not always applied to animal experimentation. When such methods are incorporated into animal studies, a treatment effect is less likely to be reported. Also, if a drug is administered in error or at the wrong dose, or if injury seems inadequate, animals typically are dropped from the study and replaced. Finally, a minority of neuroprotective agents have been evaluated by more than one research group using consistent methodologies. For these reasons, basic research on neuroprotective agents should adopt more rigorous methods, analogous to those in clinical trials, such as randomization, masking, prespecified analysis plans, and replication of findings and reproducibility of results in more than one laboratory.
- **Premature Initiation Of Clinical Trials.** It seems that clinical trials frequently are initiated with insufficient preclinical data. For example, preclinical studies rarely demonstrate long-lasting neuroprotective effects. Studies that evaluate only one time point (which may be a relatively short interval after the injury) may actually represent a delay, but not an arrest, of cell death. Assessments at later time points are necessary to prove sustained neuroprotection (Fisher M et al.,1999).

B. Issues concerning clinical trials

- **Problems In The Execution Of Neuroprotection Clinical Trials.** Clinical trials have inherent limitations. To encourage enrollment, inclusion criteria may result in discrepancies between the patient's injury and that studied in the animal model. Heterogeneous levels of injury may preclude identifying a benefit for more strictly defined and homogenous subgroup of patients. More carefully selected subgroups may be more likely to identify a benefit, but have the disadvantage of requiring a longer study time in order to recruit adequate numbers.
- **Choice Of Clinical Endpoints.** The decision of primary endpoints is critical to demonstrating that a therapeutic intervention is efficacious (Fisher et al., 2001). A major difference between animal and clinical studies is choice of outcome measures. Most animal studies use pathologic endpoints such as number of preserved retinal ganglion cell bodies or axons to classify successful treatment responses. Clinical trials of glaucoma, however, judge efficacy by using imaging or functional outcomes (visual field-VF testing), or both, most often months after the initial insult. However, VF testing is limited by the variability in results, and usually multiple tests are used to reduce the variability and increase specificity. Structural biomarkers in optic nerve and retinal disease include new technologies such as optical coherence tomography which could quantitatively evaluate RGC layers, nerve fiber layer and focal thickness, size of the neuroretinal rim, and number of RGCs or axons. Such tools may provide an endpoint

that potentially can be used in both preclinical and clinical studies. However, significant research needs to be carried out to determine how changes in these parameters correlate with visual function and whether they can be used as a surrogate for visual outcome.

4. Conclusion

Glaucoma is a multifactorial disease in which multiple factors interact to precipitate the disease. Various genes and pathways are involved in glaucoma pathophysiology. Incidentally, a recent report has identified a potential regulatory network operating in astrocyte-mediated neurotoxicity in cases of glaucomatous neurodegeneration (Nikolskaya et al., 2009). Their data on genetics, gene expression and proteomics provide a more detail network of genes involved in glaucomatous neurodegeneration and have led to the identification of some key network hubs involved in astrocytes-mediated neurotoxicity in glaucoma. Their analyses have also indicated the involvement of the immune system, oxidative stress, alteration of the extracellular matrix structure and glutamate excitotoxicity in glaucomatous neurodegeneration. Moreover, it was found that over two thirds of the genes linked to glaucoma by genetic analysis can be functionally interconnected into one epistatic network via experimentally-validated interactions. It is more obvious now that it is very hard to inhibit glaucomatous damage by inhibiting one particular pathophysiologic mechanism. It is becoming clear that novel neuroprotectants should be characterized by multiple modes of actions targeting key biological network modules. Along these lines, combinations of medications with different mechanisms of action are more likely to produce better results with fewer adverse effects. Thus, a multidrug approach may be useful, which includes agents targeted toward lowering IOP as well as an agent directed at preserving and protecting the optic nerve from different mechanisms of glaucomatous damage (targeting both cellular energy metabolism and excitotoxicity).

Tools such as new imaging technologies have been developed and may be incorporated into studies providing endpoints that potentially can be used in both preclinical and clinical studies. In this way premature initiation of clinical trials could be avoided. Furthermore, development of minimally invasive (subconjunctival injection, eyedrops) biotechnical or prolonged methods of drug delivery might increase the efficiency and safety of neuroprotectants. In addition, intense research in gene therapy has made it an emerging therapeutic possibility in glaucoma management. Intraocular transfer of genes expressing neurotrophic factors or their receptors and antiapoptotic proteins has demonstrated neuroprotective capacity *in vivo*.

Despite the challenging history of neuroprotectants in various disease states, there is a convincing rationale for the use of neuroprotection as a therapy for glaucoma. Clearly, the concept of neuroprotective agents playing a major role in glaucoma management continues to be an exciting area of research within the glaucoma field. However, a better understanding of the pathophysiological mechanisms involved in glaucoma and better designed randomized clinical trials will undoubtedly lead us to new, safe and effective neuroprotective therapy.

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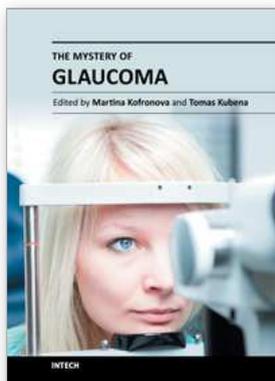
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The Mystery of Glaucoma

Edited by Dr. Tomas Kubena

ISBN 978-953-307-567-9

Hard cover, 352 pages

Publisher InTech

Published online 06, September, 2011

Published in print edition September, 2011

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.

How to reference

In order to correctly reference this scholarly work, feel free to copy and paste the following:

Eleni Bagli and George Kitsos (2011). Neuroprotective Agents in Glaucoma, The Mystery of Glaucoma, Dr. Tomas Kubena (Ed.), ISBN: 978-953-307-567-9, InTech, Available from:

<http://www.intechopen.com/books/the-mystery-of-glaucoma/neuroprotective-agents-in-glaucoma>

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