Special Focus on Glaucoma

Chapter 2

Molecular Events in Primary Open Angle Glaucoma

Goutham G[1]; Arumugam M[1]; Manikandan R*

[1]Department of Zoology, University of Madras, Guindy Campus, Chennai-600025, India

*Correspondence to: Manikandan R, Department of Zoology, University of Madras, Guindy Campus, Chennai-600025, India

Email: manikandanramar@yahoo.co.in

1. Introduction

The recurrent theme in glaucoma pathology and treatment is that elevated intraocular pressure (IOP) in most patients is the only modifiable risk emanating from defective circulation in the aqueous humor in the affected eye. Other pathological features and their unraveling mechanisms exist that remain to be fully characterized. Interestingly, the molecular events that lead to glaucoma and eventually optic nerve damage have only begun to be understood. We would like to focus extensively the development of primary open angle glaucoma (POAG) and the molecular basis in the development of this pathological manifestation. POAG is the most common of the glaucomatous types accounting nearly three-fourths of the afflicted populace [1]. Furthermore, it has been projected the number of glaucoma patients in the US to exceed 7 million in 2050 from over 2 million in 2011 with the elderly being the most affected by the disease [2]. Our contribution would focus the cellular physiology and molecular events that govern IOP that are dysregulated in pathology such as in the development of glaucomatous disease. In addition, this chapter will try addressing current research including the different signaling mechanisms and their mediators that contribute in the pathogenesis of POAG.

2. The Molecular Etiology of POAG

The micro circulation of aqueous humor (AH) is central to IOP and its bulk outflow from the anterior chamber of the eye is tightly regulated. It becomes obvious that any aberrations in the inflow or outflow of AH would result in elevated IOP that in turn leads to glaucoma. However, the complexity of the disease lies not only in characterizing the molecular pathways involved within the chambers of the eye but also those in the surrounding environment that

eventually disrupts AH circulation. The trabecular mesh (TM) framework is an integral part of AH outflow in the anterior chamber of the eye that is embedded in extracellular matrix (ECM) beams [3]. The TM cells line the endothelial spaces that drain into the Schlemm's canal (SC) and this feature is known to exert the resistance to AH outflow from the anterior chamber to modulate IOP (**Figure 1a**). While the bulk of AH is drained via the Schlemm's canal, a significant minority of the outflow has been known to exit through the extracellular spaces in the ciliary muscle. This latter route, also known as the uveoscleral pathway, is reported to account at least 3-15% of AH outflow [4] and is thought to function independent of changes in IOP. This chapter will majorly deal with the molecular events and their regulation in the Trabecular mesh framework- the Schelmm's canal pathway and its disruption in POAG pathogenesis.

As mentioned previously, POAG is the most common type of glaucoma wherein impairment in the exit of AH from the anterior chamber of the eye is known to contribute to development of disease. An increase in IOP is known to occur in most patients though normal tension glaucoma (NTG) among a population of those afflicted with POAG does exist [5]. The chronic condition of elevated pressure in the eye has been observed to result in further pathological manifestations including damage to the optic nerve head (ONH) and loss of vision. Thus glaucoma presents a formidable challenge not just in terms of diagnosing raised intraocular pressure that could very well be normal, mild or elevated but also monitoring retinal health before the disease progresses to irreversible optic nerve damage and permanent blindness. To complicate this further, other forms of glaucoma such as congenital and primary angle closure glaucoma (PACG) are also prevalent which necessitates constant monitoring for the disease, as with no reliable symptoms across the types that have been reported until now.

3. ECM Remodeling and Glaucoma Pathophysiology

A detailed understanding in the physiology of the eye's chambers that circulate and drain AH is crucial in the approach to treatment of glaucoma. It cannot be understated the importance of the Trabecular meshwork- Schlemm's canal pathway [6] in maintenance of ocular pressure and therefore a careful dissection in the functioning of TM network is paramount. The TM cells have been known to rest in layers of ECM and confine AH in intra-trabecular spaces confined within them. The TM cells form finger like extensions and are known to be phagocytic and entrap debris in the AH. The deepest layers of the TM network about the endothelium of the SC and comprise the juxtacanicular (JCT) region. Several studies report the distribution of ECM components across the TM, JCT and the SC inner wall regions including collagen fibres, proteoglycans (chondroitin sulphate CS), fibronectin and other matricellular proteins. It is interesting such ECM components are not uniformly distributed throughout the TM network but also in specific cases pinpoint regions where AH outflow varies considerably.

Such is the case that has been observed in the expression of versican, a CS proteoglycan,

in regions of lower AH outflow [7] and vice versa under physiological conditions. Another fitting illustration to the differential expression of regulators involved in maintenance of IOP is the presence of I- and B- pores with the latter found to be present in regions of higher AH drainage [8]. Such homeostatic features are a target in pathological conditions such as in the development of glaucoma where in ECM remodeling has been reported to be extensively involved in disease progression. Several studies have investigated the role of ECM remodeling in the contexts of organ development, wound healing and disease. The elasticity of the tissue extracellular matrix is important for AH outflow [9] that has been known to be altered in glaucomatous patients owing majorly to the expression of matrix metalloproteases (MMPs). Further, activation of predominant isoform of the transforming growth factor 2 (TGFβ2), an important cytokine that has been extensively characterized in tissue fibrosis, in the TM of eyes afflicted with glaucoma [10] has been known to affect various pathophysiological functions including cell adhesion, cytoskeletal modification and extensive ECM reorganization.

It is precisely for aforesaid reasons the activation of TGFβ2 is tightly regulated. The induction of smad signaling, activation of RhoA GTPases, inflammatory and cellular stress mediators by TGFβ also work in tandem with bone morphogenetic protein (BMP) signaling with several important consequences downstream. The upregulation in connective tissue growth factor CTGF [11] has been known to remodel the ECM extensively. The levels of MMP-2, α- smooth muscle actin, TNF-α among other pro-inflammatory cytokines correlate positively with glaucoma as with the decreased synthesis of the glycosaminoglycan (GAG) hyaluronan that reduced TM outflow to overall contribute to elevated IOP [12]. The RhoA GTPase deserves a special mention owing to its mediation via the downstream effector ROCK (Rho associated serine threonine kinase) that in turn regulates cell contractility and ECM synthesis via phosphorylating substrates including myosin light chain (MLC) to regulate focal adhesions and actin stress fiber formation [13]. The Rho kinase inhibitors have garnered interest due to their ability to dampen TGFβ2 activation and TM cell trans-differentiation, in addition stimulate endothelial nitric oxide synthase (eNOS) that promotes vasodilation and ECM relaxation [14] to eventually restore homeostatic levels IOP. Zhang and Rao [15] reported the compound blebbistatin improved AH outflow by inhibiting myosin ATPase activity.

4. POAG and Oxidative Stress

It cannot be disputed the raise in IOP leads to glaucoma and the progression of which when left unchecked leads to retinal damage and eventually loss of vision. Elevated fluid pressure has been well documented to alteration in TM compression [16] and cellular damage. Several reports attest to increased levels of free radicals and DNA damage concomitant with depleted anti-oxidant levels and POAG [17]. As a consequence, the activation of major cellular stress pathways including the nuclear factor-κB (NF- κB), mitogen activated protein kinase (MAPK) and cell death (via the induction of caspase-3) [18] have been implicated in

glaucoma. Intriguingly, mild but chronic conditions of vascular deficiency has been postulated as a means that not only perturbs physiological functioning of TM network but also the real possibility of ischemia and reperfusion mediated injury that amplifies the production of free radicals including the highly reactive and most damaging peroxynitrite ($ONOO^-$) anion. Mechanisms including mitochondrial dysfunction, accumulation of ECM components in the anterior chamber of the eye and impaired blood contribute to disease development and progression. Oxidant mediated damage in particular has been implicated in pseudo exfoliation glaucoma (PEX) [19] and numerous markers indicating oxidative insult to the eye have been reported such as lipid peroxidation products, DNA strand breaks and depleted cellular reserves of anti-oxidant enzymes.

A major target in the imbalance of cellular oxidants and anti-oxidant defenses is the mitochondrial functioning that has been implicated in many systemic diseases involving vascular deregulation, arterial hypotension and arteriosclerosis. Systemic ischemic conditions arising from the above conditions can fuel mitochondrial dysfunction leading to bioenergetic failure [20] and worsen oxidative stress mediated mechanisms that steer cells towards activation of apoptosis. In addition to elevated IOP, aging is positively correlated with increased risk for ONH damage [21]. It is not difficult to comprehend oxidative stress mediated damage, both as a cause and a consequence to disruption in mitochondrial functioning, has a definite role to play in glaucoma [22]. Studies highlight oxidative stress markers such as heme oxygenase-1 (HO-1), the lipid peroxidation products including 4-hydroxy noneal (4-HNE) [23,24] that are upregulated in glaucoma. An emerging interplay between mitochondrial biogenesis and calorie restriction [25] has been unraveling that is vital in promoting organelle and ultimately longevity at the level of the organism. We [26] have also reviewed this recently wherein resveratrol administration leads to activation of molecular pathways that combat inflammation and promote cellular homeostasis especially in the context of preventing and managing outcomes to ocular diseases.

5. Neuroinflammation and Glaucoma

Inflammation is a near ubiquitous event that is both essential in normal cellular physiology but is also implicated in a range of pathological conditions from fibrosis, cancer and inflammatory disease. In all its complexity involving different neuro-inflammatory processes and cellular players, what makes it intriguing is glaucoma has been reported to induce both by ongoing inflammatory processes and also treatments, as with glucocorticoid treatments to alleviate inflammation [27]. As we have discussed above, distinct yet interconnected events occurring at two ocular sites, namely the TM network at the anterior chamber of the eye that drains AH and the optic nerve head beyond the vitreous through the lamina cribrosa region (unmyelinated axons of RGCs pass through openings in a plate like structure made of collagen) contribute [28] to glaucoma pathology (**Figure 1b**). Alongside the cellular processes that oc-

cur at different sites leading to the disease, the key players and their associated mediators also need a careful investigation to arrive at a holistic view of pathological features and treatment options available to us. The lamina cribrosa (LC) is especially important since many features associated with the functioning of ONH have been found to be affected in glaucoma. Several pathological hallmarks of the disease that include the cupping of ONH and thickening of the ECM that indicate axonal compression and impaired cellular transport and more importantly astrogliosis have all been reported [29].

Astrocytes are the principal component cell type that provides structural support to neurons. They can be ascribed functions akin to the retinal pigment epithelium is to the photoreceptor cells of the retina. Their role becomes evident when problems arise, as observed with astrogliosis, a pathological condition that manifests especially in POAG. Type 1 astrocytes are the dominant type that serves the unmyelinated axons in the pre laminal and laminar regions of the optic nerve [30]. In a further classification, the type 1B astrocytes along with LC cells form the LC in the ONH. These resting cells are activated under conditions of cellular stress involving changes in morphology, altered patterns in secretion of ECM and axonal cell body hypertrophy [31]. The thickening in ECM is a major characteristic feature in glaucoma pathology indicating ONH damage that is brought about by astroglial reactivation [32]. The activation of different cellular mediators such as TGFβ, MMPs and others that exacerbate disease symptoms have been discussed already in this chapter. While the molecular mechanisms of different mediators involved and their contribution to pathology is relatively well understood, a few challenges remain: the interplay between astrocyte activation to changes in anatomical features of the ONH; validating markers involved in disease progression involving RGC death that results from astrogliosis (especially since the activation, at least in part, has been known to be beneficial as in the case with optic nerve injury and repair); and finally, the search for a suitable animal model that closely mimics pathological features of human glaucoma besides investigate different genetic knock-out models for a better understanding of the disease.

6. Cellular Proteostasis and Glaucoma

Under conditions of prolonged cellular stress, the induction of the unfolded protein response (UPR) triggers activation of pathways-the ubiquitin- proteasome system (UPS) and autophagy-that attempt to redress the imbalance in protein homeostasis. The role of protein degradation and its impairment in the development of various metabolic disorders and neuro degeneration such as type 2 diabetes (T2D), Alzheimer's and Parkinson's diseases is emerging and a few recent observations have reported the inhibition of such pathways to detrimental consequences. This is very relevant in the pathology of glaucoma because many investigations reveal misfolding and aggregation of myocilin, a glycoprotein that is expressed in both ocular and non-ocular tissues [33]. Suntharalingam *et al.* [34] for instance underscored the importance of the endoplasmic reticulum associated degradation pathway (ERAD) and autophagic

clearance of aggregated myocilin *in vitro.*

The TM cells much like the retinal pigment epithelium underlying the retina are quiescent cells and entrap debris to help regulate AH outflow. It is very conceivable the phagocytic activity is very essential and constantly functions in protein turnover via the autophagic pathway. It is known that autophagy occurs at a very low level constitutively and the different forms of autophagy occur both in normal and pathological conditions that process intracellular protein aggregates from debris to intact organelles. A rodent model of induced ischemia was conducted [35] to determine levels of LC3II and Beclin-1 (that promote autophagosome formation). It was observed the increase in IOP was also associated with reduction in levels of the aforesaid markers that demonstrates the beneficial role of autophagy in ocular homeostasis. A protective effect in mitochondrial functioning was also observed by Want *et al.* [36] when the autophagic pathway was not inhibited. However, it must be emphasized autophagic induction, depending on cellular contexts and progression of the disease, can have negative consequences to stress and survival including activation of cell death [37]. It is important to bring our attention to cases in which aging and mounting conditions of cellular stress such as elevated generation of reactive oxygen species (ROS) that perhaps mild but chronic in nature might impair lysosomal degradation via autophagy and predispose to disease [38]. This could very well be relevant in the development of POAG and requires a thorough investigation. A schematic representation featuring RGC death owing to chronic elevated IOP and its associated pathways has been depicted in **Figure 2.**

7. Current Challenges and Future Directions

One cannot overstate the importance of understanding the molecular basis of glaucoma in order to devise effective strategies in diagnosis and treatment. Two prominent theories in relation to pathogenesis of the disease will be addressed here to attempt incorporating several molecular features and signaling pathways in each of them. While both seem plausible it can be said that molecular events that play out from each theorized model do not act independently from one another but rather in concert to elevate IOP in most glaucoma cases and also cause ONH damage especially in the later stages of the disease. The biomechanical theory attributes the changes in the structural framework of the eye to disease formation. Strouthidis and Girard [39] posit any change to the LC region in the ONH is fundamental in influencing the pathophysiology of the eye. It is understandable the site of the optic nerve exiting the eye is mechanically weaker that is susceptible to stress or strain forces that in turn is capable of inducing profound changes including compression of the axonal fibres in the optic nerve leading to RGC stress and death. What follows this is the characteristic cupping of the ONH and the activation of astrocytes that can exacerbate cellular stress via mediating extensive ECM remodeling, disrupting RGC homeostasis and raising IOP. In regards to devising novel therapies in treatment of glaucoma via altering the biomechanics at the lamina cribrosa region and

the peripapillary sclera surrounding the ONH, experiments were undertaken both ex vivo and in animal models to attempt increase sclera thickening as a means to minimize cellular damage from chronic IOP elevation [40]. However, concerns regarding safety and the effectiveness of such interventions need to be addressed. It should be interesting to observe the response of neurons and astroglial cells to invasive peripapillary scleral stiffening and other structural changes in the eye.

Vascular dysregulation highlights the impairment in regulating blood flow to the affected region in light of changes in perfusion pressure such as the case with increased IOP in glaucoma patients. Reports exist of alterations in ocular blood flow in patients suffering from POAG [41,42]. This failure in autoregulation of blood flow in an organ further compounds the problem via promoting ischemia and oxidative stress insult via increasing the production of ROS. Again, it has been observed patients with glaucoma exhibit in their AH diminished anti-oxidant defense levels [43] and heightened levels of lipid peroxidation products such as malondialdehyde [44]. It therefore is imperative to develop therapeutic agents that can be administered to patients and high risk groups (such as those with familial history or elderly people for instance) with the ability to mitigate RGC stress and death independent of IOP. Of equal importance will be to utilize study such therapeutic agents in both clinical trials and relevant models of disease for reliable assessment of outcomes to such potential targeted therapies. Currently available drugs for glaucoma treatment such as β-adrenergic receptor blockers that decrease AH inflow, the cholinergic class of drugs that act in opposite manner via increasing outflow via the TM network and the prostaglandin analogs, which modulate MMP activity, among others chiefly target to relieve IOP [45]. While such drugs remain the mainstay treatment, novel IOP lowering medication and drugs that target other pathological molecular processes such as oxidative stress and neuroprotective agents that regulate cellular homeostasis also need to be explored.

Another exciting area of interest has been to identify the genes that regulate IOP and TM cell functioning that when mutated, as observed with glaucoma patients, leads to formation of the disease. Myocilin (MYOC) has been a major focus of study among different experimental groups that, in addition to being mutated in those with POAG [46], was observed to mediate detrimental effects in animal and in vitro models of study, when over-expressed. MYOC is a secreted glycoprotein that is present in both in ocular and non-ocular tissue. Although its physiological role is not very certain, it is possibly involved in axonal myelination [47]. A minority of POAG patients [48] were found to express misfolded MYOC protein aggregates that contributed to pathology. Mutations in the olfactomedin (OLF) domain have been implicated in severe form of the disease [49]. A few investigations wherein the accumulation of the misfolded protein and the modulation of downstream signaling pathways have been reviewed in brief here. Using a transgenic model to study the fate of mutated MYOC in fruit flies, Car-

bone *et al.* [50] revealed the discharge of AH, elevation in intraocular pressure, and activation of the unfolded protein response. In vitro studies were also carried out to investigate myocilin aggregation in a human embryonic kidney cell line (HEK 293T) model which demonstrated a mutant form of MYOC was resistant to cleavage by calpain II (a calcium dependent protease) and prone to aggregation especially during conditions of oxidative stress [51]. The endoplasmic reticulum (ER) is the major cellular organelle involved in post-translational modification of newly synthesized proteins. Any disturbance in the normal functioning of the ER has been known to induce cellular stress and even translational arrest. The ERAD pathway is then activated to help clear misfolded and aggregated proteins to alleviate the ER stress load. However, under conditions that impair the ERAD pathway, autophagic degradation of cellular debris can be activated that possibly promotes efficient clearance of such aggregated proteins. This mechanism was demonstrated with a mutated form of myoclin *in vitro* [34] and hence underscores the need to decipher autophagy mediated mechanisms relevant to treating glaucoma. A few other genes such as optineruin (OPTN) and TANK binding kinase 1 (TBK1) have also been studied via family based genetic linkage analysis to detect allelic variations that influence POAG [52]. Yet, much work still remains to be accomplished including characterizing molecular pathways that contribute to glaucoma. It is important the multiple avenues of research in the etiology, propagation and outcomes to the different sub-types of the disease be pursued concurrently and the seemingly disparate pieces of information from such studies can help in strategizing steps in disease mitigation and possibly even prevention.
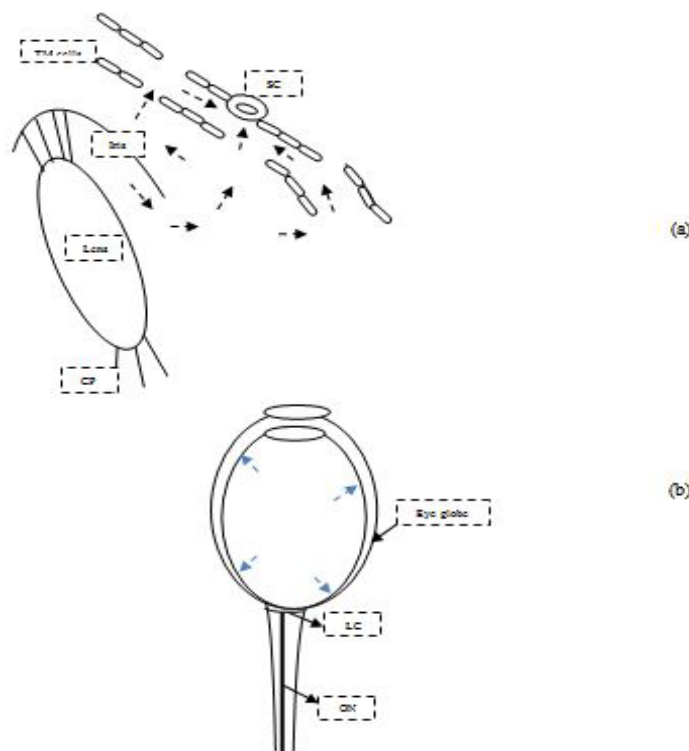
8. Figures

Figure 1: Simplified illustration of (a) the anterior eye chamber with the Trabecular mesh **TM** cells draining the aqueous humor **AH** (indicates direction of AH flow) via the Schlemm's canal **SC** with the lens held by ciliary process **CP** in the posterior chamber (modified from Sowden 2007); (b) the eye globe with the lamina cribrosa **LN** and the optic nerve **ON** at the posterior end (indicates intraocular pressure; modified from Wang *et al.* 2016)
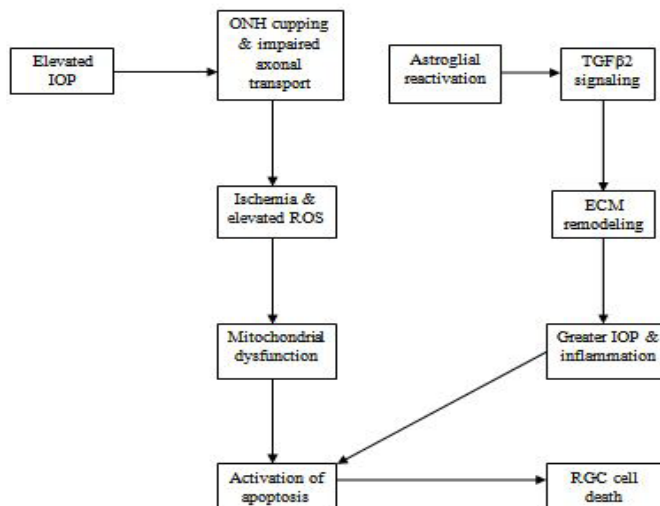
Figure 2: Schematic representation of retinal ganglion cell death in POAG and associated pathways.

9. References

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