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Genetics and Environmental Stress Factor Contributions to Anterior Segment Malformations and Glaucoma

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<http://dx.doi.org/10.5772/54653>

1. Introduction

Glaucoma is one of the leading causes of irreversible blindness worldwide [1]. A gradual loss of retinal ganglion cells (RGCs) result in degeneration of the optic nerve head and visual field loss. Glaucoma is an age-related disease with a strong genetic basis. The risk of developing glaucoma significantly increases after age 40 [2,3]. An estimated 79.6 million people worldwide will have glaucoma by 2020 [1]. Patients with mutations in glaucoma-associated genes are more likely to develop juvenile-onset and early adult-onset glaucoma. In any case, early detection of glaucoma is essential to effectively manage the progression of the disease by preventing further loss of RGCs. Despite many years of research in this field, the precise cause(s) of RGC death remain unknown. The pathophysiology of glaucoma is complicated as environmental, genetic, and even stochastic factors all contribute to the pathology of glaucoma. Also, both the posterior segment, where the RGCs are located, and the anterior segment of the eye play key roles in the disease.

Glaucoma can be classified as being primary, secondary, or congenital. These groups can then be further categorized to be open-angle or closed-angle, depending on the anterior chamber angle. In closed-angle glaucoma, the angle between the iris and the cornea is closed resulting in obstruction of aqueous humor flow. Primary glaucoma is non-syndromic and is not associated with any underlying condition. Primary congenital glaucoma is a rare form of glaucoma present at birth or within the first two years after birth. Glaucoma that develops as a result of an underlying ocular or systemic condition or eye injury is categorized as secondary glaucoma. Pseudoexfoliative glaucoma is an example of secondary glaucoma whereby fibrillar

extracellular material deposits and accumulates in various ocular tissues, predisposing the patient to developing glaucoma.

Primary open angle glaucoma (POAG) is a common type of glaucoma where the iridocorneal angle is unobstructed. Although POAG can occur in patients with normal intraocular pressure (IOP), sometimes referred to as normal-tension glaucoma, elevated IOP is a major risk factor of developing POAG. IOP is dependent on proper flow of aqueous humor from the site of production in the posterior chamber to the site of drainage in the anterior chamber of the eye. The anterior chamber structures that function in regulating the drainage of aqueous humor from the eye are the trabecular meshwork (TM) and Schlemm's canal. Disruptions of the aqueous humor flow pathway are predicted to result in elevated IOP.

In this chapter, the recent advances in research regarding the contribution of the TM in maintaining proper IOP will be reviewed. An overview of the anterior chamber drainage structures, the TM and Schlemm's canal, and how these structures maintain the aqueous humor outflow pathway will be provided. Also, the changes that occur in the TM during the normal aging process and in the glaucoma phenotype will be compared. Then, the specific types of stresses that TM cells are exposed to, mainly mechanical, oxidative, and phagocytic stresses, and the effects these stresses have on gene expression will be examined. Recent advances in technology have enabled the analysis of global gene expression profiles. These analyses have revealed that signal transduction pathways play an important role in the cellular adaptive response to environmental stresses. Finally, the effect that environmental stresses have on glaucoma-associated genes will be considered.

2. Trabecular meshwork and aqueous humor outflow pathway

Aqueous humor is a colourless and transparent fluid that makes contact with various structures in both the anterior and posterior chambers of the eye including the lens, iris, and cornea. The lens and the cornea are clear and avascular, which enables light to be effectively transmitted to the photoreceptors in the back of the eye. Aqueous humor provides nutrients to the avascular lens and cornea and also removes metabolic waste products. The composition of aqueous humor has been of great interest due to the potential regulatory effects on all the structures to which it makes contact. For example, the presence of antioxidants such as glutathione and ascorbic acid [4,5] in the aqueous humor suggest that this fluid affects the ability of cells to respond and adapt to stress.

Aqueous humor flows from the site of production, which is the non-pigmented ciliary epithelial cells [6,7] in the posterior chamber, to the site of drainage, which is the TM and Schlemm's canal in the anterior chamber (Figure 1). Production and drainage of aqueous humor is a continuous and dynamic process. Diurnal variations in aqueous humor turnover rates occur ranging from 3.0 μ L/min in the morning to 1.5 μ L/min at night [8]. The balance between aqueous humor production and drainage is essential for maintaining a healthy IOP of approximately 15mmHg within the eye [9]. Abnormalities in aqueous humor drainage due

to increased resistance at the TM are thought to result in elevated IOP, which is a major risk factor for developing glaucoma [10].

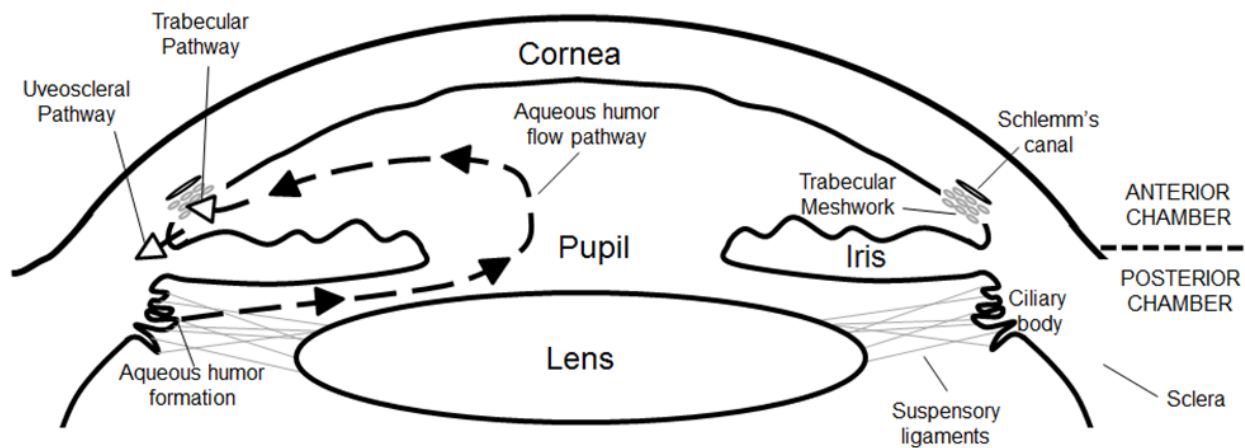


Figure 1. Schematic diagram of aqueous humor flow pathway. Aqueous humor is produced by the ciliary body in the posterior chamber and then flows into the anterior chamber. The majority of the aqueous humor will be drained from the eye via the trabecular pathway through the trabecular meshwork (TM) and Schlemm's canal. The rest of the aqueous humor is drained via the uveoscleral pathway. Increased resistance occurs when the TM and Schlemm's canal malfunction. This disruption in aqueous humor outflow leads to increased intraocular pressure (IOP), which is a major risk factor for developing glaucoma.

Aqueous humor is drained from the eye by two distinct outflow pathways: the trabecular (aka conventional) pathway and the uveoscleral (aka unconventional) pathway. The uveoscleral pathway is an IOP-independent pathway in which the aqueous humor leaves the anterior chamber by passing through the ciliary muscle bundles into the supraciliary and suprachoroidal spaces and eventually into the sclera [11,12]. Direct measurement of the percentage of aqueous humor leaving the human eye via the uveoscleral pathway has proven to be difficult [13]. There appears to be great variation between individuals with values ranging from 36% to 54% in healthy young subjects [14,15]. The percentage of aqueous humor leaving the eye via the uveoscleral pathway decreases with age with values ranging from 4% to 46% in older subjects [15,16]. Thus, as aging progresses, a larger portion of aqueous humor is drained via the trabecular pathway.

Despite the individual variations, it is generally accepted that in humans, the majority of aqueous humor is transported through the TM via the trabecular pathway. Disruption of aqueous humor drainage through the trabecular pathway is thought to be the major contributing factor to alteration of IOP. The TM is a multi-layered tissue located in the anterior chamber angle. From the anterior chamber the aqueous humor passes through the multiple layers of the TM: the uveal meshwork, the corneoscleral meshwork, and the juxtacanalicular meshwork (also known as the cribriform plexus). Each layer consists of a central connective tissue (aka beam) surrounded by an outer endothelial layer. Connecting fibrils tightly connect

the network of elastic fibres in the juxtacanalicular meshwork to the inner endothelial wall of Schlemm's canal [17-20]. As the aqueous humor passes through each layer of the TM, the intercellular space narrows resulting in increased resistance. Then, aqueous humor progresses through the inner endothelial cell layer of Schlemm's canal. The endothelial cells of Schlemm's canal express the tight junction protein Zona occludens-1 (ZO-1), which allows aqueous humor to be transported via the intercellular route [21]. The aqueous humor is also transported via the transcellular route through giant vacuoles [22-24]. Aqueous humor passes through Schlemm's canal and returns to the general circulation via the aqueous and episcleral veins [23,25]. IOP is affected by the episcleral venous pressure and the resistance to aqueous humor flow within the TM. Episcleral venous pressure directly affects IOP because aqueous humor must flow out of the eye against the pressure in the episcleral veins. The main source of resistance to aqueous humor flow is thought to be located in the intercellular (aka subendothelial) region of the juxtacanalicular network [26-29].

Extracellular matrix (ECM) occupies the intercellular space between the beams of TM cells. The ECM consists of glycosaminoglycans (GAGs), proteoglycans, laminin, various collagens, fibronectin, and vitronectin (reviewed in [30]). The constant turnover of this ECM has been proposed to play a role in maintaining proper aqueous humor resistance. The family of matrix metalloproteinases (MMPs) are secreted zinc proteinases that initiate ECM turnover [31,32]. MMP activity is inhibited by the family of tissue inhibitors of metalloproteinases (TIMPs). MMP activity is suggested to be important in regulating aqueous humor outflow facility by proteolytic alterations. Using perfused human anterior segment, Bradley *et al.* observed that increasing MMP activity increased the outflow rate while inhibiting MMP activity by the addition of TIMP decreased outflow rate [32]. MMP activity is suggested to have various functional consequences including degradation of ECM components, cleavage and modification of signaling molecules, and cleavage of intercellular junctions and basement membrane (reviewed in [33]).

Another factor that affects resistance is the ciliary muscle. The elastic anterior tendons of the ciliary muscle insert into the network of elastic fibres in the juxtacanalicular meshwork and corneoscleral meshwork [19,20,34]. The elastic fibres are surrounded by a collagen-based sheath [20]. Ciliary muscle contractions result in increased aqueous humor outflow facility [35]. Upon ciliary muscle contraction, the connecting fibres straighten. Since the ciliary muscle is connected to the TM and the inner wall of Schlemm's canal by the connecting fibrils, ciliary muscle contraction widens the intercellular space in the juxtacanalicular meshwork allowing aqueous humor to flow against less resistance [35]. In contrast, relaxation of the ciliary muscles results in the opposite effect where there is increased resistance to aqueous flow [36].

As outlined above, the aqueous humor flow pathway is a complex process regulated by structures in both the posterior and anterior chambers of the eye. The TM is a highly specialized tissue that is able to adapt to the dynamic nature of aqueous humor outflow. The ability to adapt is an essential characteristic of the TM, especially because these cells are located in an environment that is constantly changing (Figure 2).

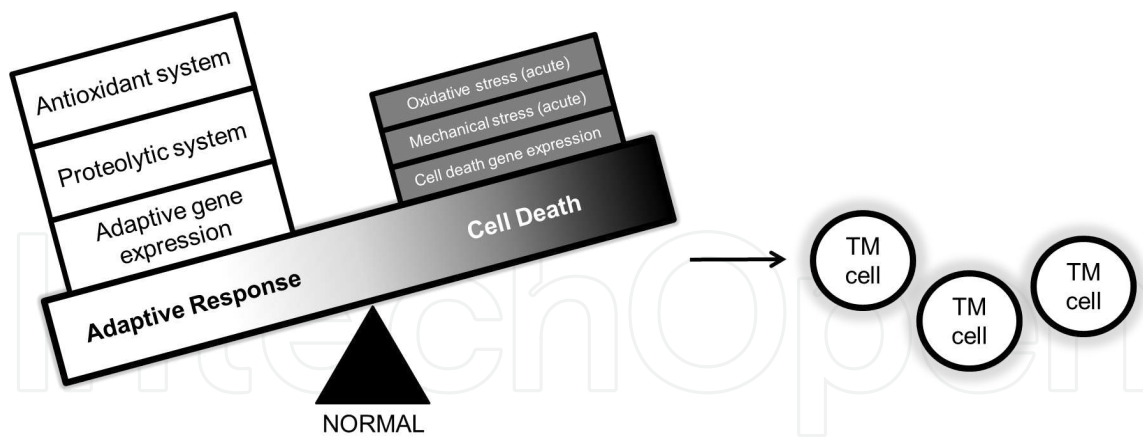


Figure 2. Trabecular meshwork under normal conditions. Even under normal conditions, the cells of the trabecular meshwork (TM) are constantly exposed to mechanical and oxidative stresses. TM cells have defense mechanisms including the antioxidant and proteolytic systems to protect the cells from these stresses. Also, specific changes in global gene expression occur in response to the specific stress, which enables TM cells to adapt to the environment and survive.

3. Change in trabecular meshwork during the normal aging process

Aging is a major risk factor for developing glaucoma. However, at the physiological level, minimal changes in aqueous humor flow dynamics occur in normal healthy subjects as aging progresses (reviewed in reference [37]). Using tonography, many studies have observed that aqueous humor outflow facility decreases with age [15,38-40]. The tonographic procedure measures outflow facility. IOP is first increased by applying force to the cornea using a tonometer probe. The subsequent decrease in IOP over the time of the test is used to determine aqueous humor outflow via the trabecular pathway. However, interpretation of results using the tonographic technique is limited because ocular rigidity is not taken into account. Since ocular rigidity increases with age [39,41], older subjects may appear to have a reduction in aqueous humor outflow facility because the stiffer eyes are less responsive to the tonographic technique, which involves applying force to the cornea. Also, the tonographic measurements do not take into account the change in pseudofacility, which refers to the probe-induced change in aqueous humor flow into the anterior chamber. In contrast to tonography, fluorophotometry is not affected by ocular rigidity and pseudofacility because no force is applied to the cornea. The outflow facility measured by fluorophotometry was $0.23 \pm 0.10 \mu\text{L}/\text{min}/\text{mmHg}$ in 20-30 year old subjects ($n=51$) and $0.27 \pm 0.13 \mu\text{L}/\text{min}/\text{mmHg}$ in subjects 60 years and older ($n=53$) [15]. Thus, fluorophotometric measurements indicate that there is in fact no difference in outflow facility as aging progresses [15]. Many studies using tonographic and fluorophotometric measurements have consistently shown that with age, aqueous humor production decreases [15,38,39,42-44]. Although outflow facility remains stable and aqueous humor production decreases, IOP remains stable in normal healthy subjects as aging progresses. Toris *et al.* have recently measured IOP to be $14.7 \pm 2.5 \text{ mmHg}$ in 20-30 year old subjects ($n=51$) and $14.3 \pm 2.6 \text{ mmHg}$ in subjects 60 years and older ($n=53$) [15]. A decrease in anterior chamber depth [15,45,46] with aging may account for the lack of change in IOP.

Interestingly, prominent changes at the structural and cellular levels occur with age. Connecting fibrils ensure that contact is maintained between the juxtacanalicular meshwork and the inner endothelial wall of Schlemm’s canal [19,20]. The sheath surrounding these elastic fibres thickens with age [47]. The intercellular space narrows due to an increase in the amount of extracellular material from the thickened sheath, resulting in increased resistance [48,49]. Also, as aging progresses, the number of TM cells decrease [50,51]. Grierson and Howes estimate that at age 20, there are approximately 763 000 cells in the TM. By age 80, approximately 403 000 cells remain [51]. The outer TM layers lose more TM cells while the least number of TM cells are lost from the inner juxtacanalicular layer [51,52]. This decline in TM cells appears to be a continuous and linear process with an estimated 0.58% loss of cells annually [50,52]. The linear decrease in TM cellularity is intriguing because the mechanism of cell loss may be different between the ages [52]. Age-related mechanisms such as accumulation of reactive oxygen species (ROS) and misfolded proteins are likely to contribute to cell loss in older subjects. However, other non-age-related mechanisms, such as exposure to mechanical stress, are likely responsible for cell death in the TM in younger subjects. Interestingly, Alvarado *et al.* noted that TM cells may have a reduced reparative capacity, which would further contribute to the decreased cellularity with age [52]. The loss of TM cells with age could have a more severe consequence in some individuals because there appears to be great variation in the absolute number of TM cells between individuals [53]. Therefore, individuals with less TM cells would be predicted to be less efficient in fulfilling the function of TM cells (Figure 3).

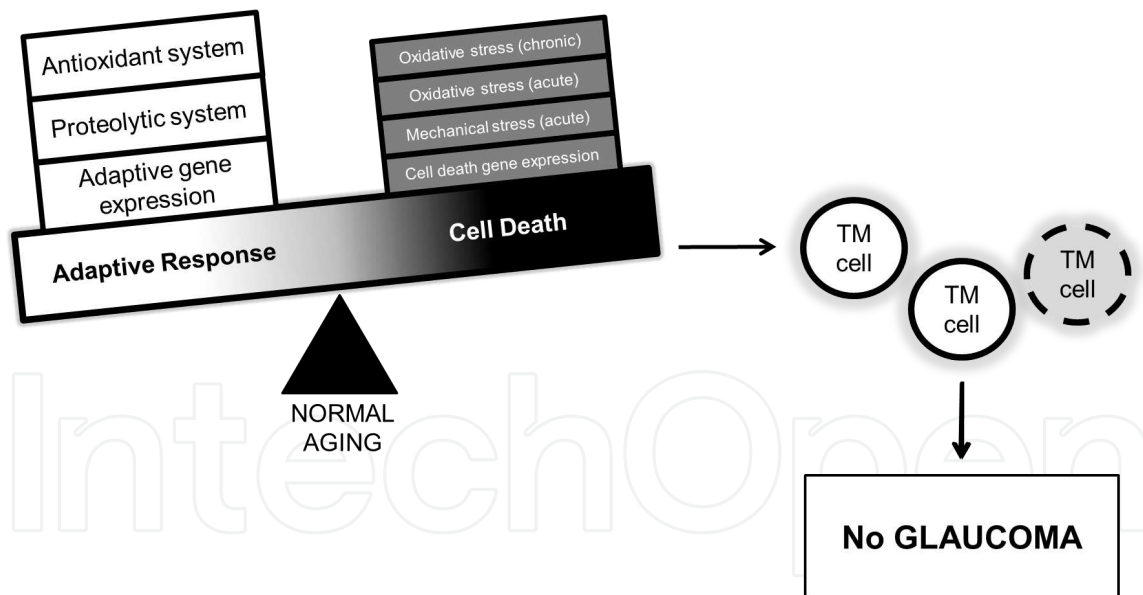


Figure 3. Trabecular meshwork during normal aging. During normal aging, the cellular defense mechanisms of the trabecular meshwork (TM) cells become less efficient. As in normal conditions (see Figure 2), the TM cells are exposed to a variety of stresses. However, the TM cells will also be exposed to other types of stresses such as chronic oxidative stress because there is an accumulation of reactive oxygen species (ROS) as aging progresses. Since the TM cells are no longer able to adapt to the environment, there will be increased TM cell death (dotted circle). However, the TM tissue still functions, preventing the onset of glaucoma.

Regardless of the individual variation in TM cell number, the consequence of losing TM cells in all aging individuals can be predicted. As avid phagocytes, TM cells are thought to clear

debris from the aqueous humor outflow pathway [54-58]]. Although TM cells have the ability to ingest particulate matters rapidly, the phagocytic process may have detrimental effects on the overall health of the cell, even leading to necrosis [59]. Zhou *et al.* also showed that after phagocytosis, temporary alteration of TM cells occurred including rearrangement of the cytoskeleton and increased migratory activity [60]. These alterations, although temporary, have been speculated to be linked to the age-related loss of TM cells [60]. TM cells also maintain aqueous humor outflow by releasing factors that regulate permeability of the endothelial cells of Schlemm's canal [61]. TM cells release various enzymes and cytokines both in the presence and absence of stimulation such as mechanical stretching and exposure to pro-inflammatory cytokines [61,62]. TM endothelial cells constitutively secrete cytokines such as Interleukin 8 (IL8), Chemokine, CXC motif, ligand 6 (CXCL6), and Monocyte chemotactic protein 1 (MCP1), strengthening the notion that the release of cytokines is important in maintaining aqueous humor outflow [62].

4. Change in trabecular meshwork in glaucoma disease phenotype

Even with the age-related structural and cellular changes, the TM effectively functions to drain aqueous humor. However, in patients with glaucoma, the structural and cellular changes are more pronounced and as a result, TM function is disrupted. In glaucomatous eyes, there is more prominent and irregular thickening of the sheaths of the elastic fibers. Also, there is increased deposition of sheath-derived plaques compared with normal eyes [47,63]. This increase in extracellular material in the TM is predicted to block aqueous humor outflow [20] contributing to the development of disease. As in normal aging, there is a linear decrease in cellularity as aging progresses in the TM of POAG patients. Moreover, Alvarado *et al.* observed fewer cells in the glaucomatous TM compared with the non-glaucomatous TM over a wide range of ages [50].

The risk of developing glaucoma significantly increases after age 40. Despite the fact that glaucoma is an age-related disease, aging in most people does not result in this disease (Figure 3). The changes that occur in the TM during the normal aging process may make the tissue more susceptible to malfunction. However, other unknown factors and even stochastic factors must be present for the TM to fail to a point that the glaucoma phenotype develops (Figure 4).

5. Exposure of trabecular meshwork to mechanical stress

In order to survive, TM cells must be able to constantly adapt to their continuously changing environment. Similar to any other cell in the body, TM cells are exposed to a variety of environmental stresses. Due to the location of cells of the TM, one of the major types of stress these cells are exposed to is mechanical stress. IOP continuously fluctuates throughout the day with a higher IOP occurring during the nocturnal period. The fluctuation in IOP is part of a normal physiological process and is unavoidable. Fluctuations in IOP occur with blinking, eye

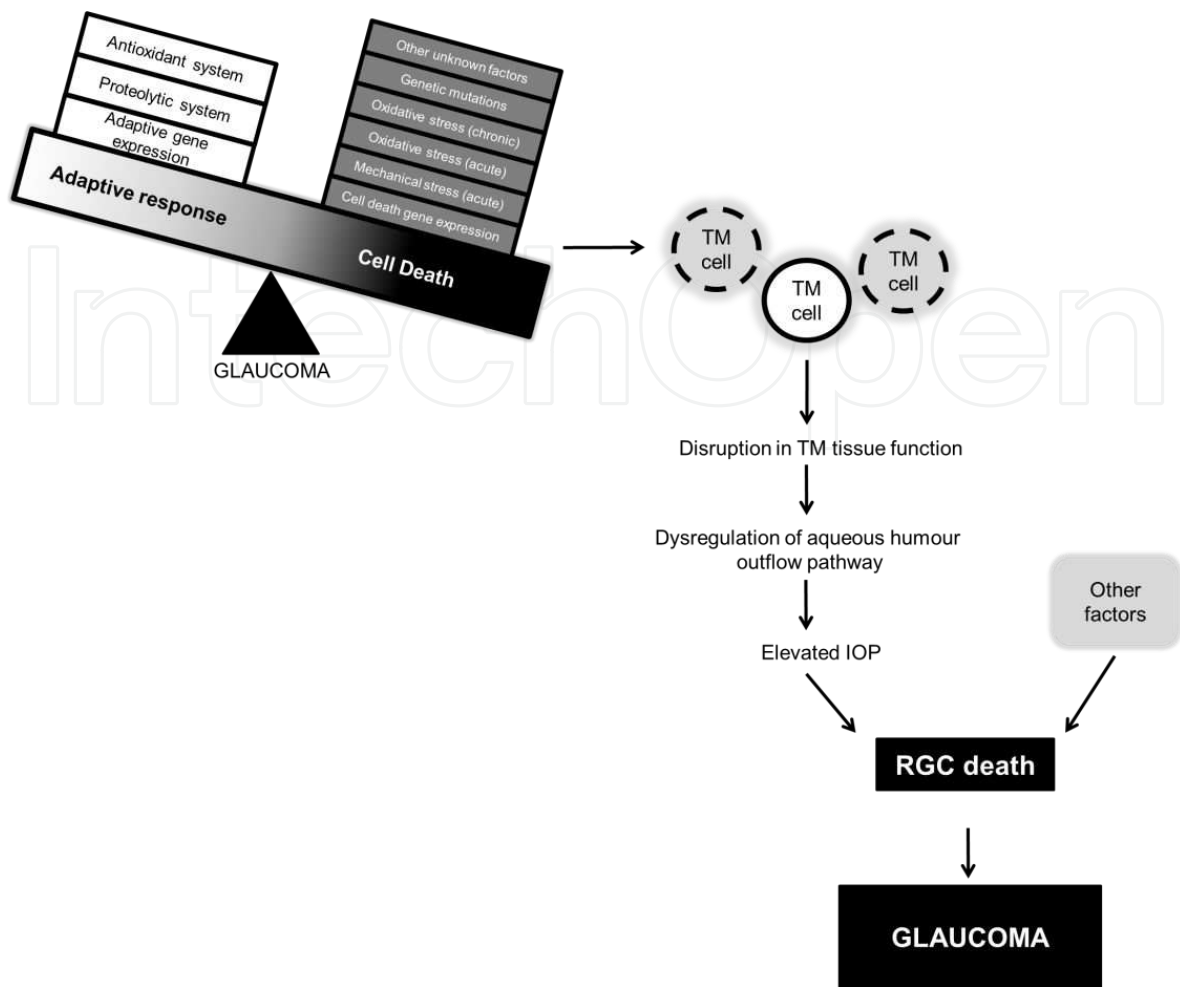


Figure 4. Trabecular meshwork of glaucoma phenotype. Similar to normal non-aging and aging conditions, trabecular meshwork (TM) cells are exposed to a variety of stresses. However, other unknown factors are present to initiate the cascade of events that lead to the development of glaucoma. Also, genetic mutations could compromise normal TM cell function. All of these factors are predicted to result in TM cell death (dotted circles) to the extent that the TM tissue is no longer able to function properly. Consequently, there will be dysregulation of aqueous humor drainage resulting in increased intraocular pressure (IOP), which would ultimately lead to retinal ganglion cell (RGC) death and glaucoma.

movements, and even with a change in body position. A supine body position has been shown to result in higher IOP compared with an upright body position [64,65]. The temporary fluctuation in IOP can vary up to 10mmHg [66]. This change in IOP results in distortions (including stretching and compression) of the cells and is sensed by the cells of the TM as mechanical stress.

6. Exposure of trabecular meshwork to oxidative stress

Another type of environmental stress that TM cells are exposed to is oxidative stress. Cells are constantly exposed to free radicals that are the by-products of normal cellular metabolism. In addition, the aqueous humor is itself a source of free radicals. Hydrogen peroxide (H_2O_2) is

normally present in the aqueous humor and is suggested to be the key source of oxidative stress for the TM [67]. Initially, the concentration of H_2O_2 , a reactive oxygen species (ROS), was reported to be between 25-60 μM in the aqueous humor using the dichloropheno-indopheno (DCPIP) assay [5,68-70]. However, technical issues with the DCPIP method, including the interference of ascorbic acid with the assay [70] and the spontaneous auto-oxidization of DCPIP in the presence of oxygen [71], has resulted in the re-examination of H_2O_2 in the aqueous humor. Different methods have indicated that H_2O_2 is present in the aqueous humor, but at much lower concentrations than previously thought [70,71]. An accurate concentration of H_2O_2 is still difficult to obtain and may vary greatly between individuals. Since cells of the TM are in direct contact with aqueous humor, these cells are exposed both intracellularly and extracellularly to oxidative stress.

Free radicals at lower concentrations are beneficial to the cell (reviewed in [72,73]). Low concentrations of ROS act as second messengers for signal transduction and gene regulation. For example, low concentrations of ROS activate the Nuclear factor kappa-B (NF- κB) transcription factor, which plays a key role in many cellular processes including inflammation, cell proliferation, and apoptosis (reviewed in [74]). However, higher concentrations of free radicals can have negative effects on the cell (Figure 5). Free radicals can damage proteins and DNA, promote lipid peroxidation, disrupt mitochondrial function, and trigger cell death (reviewed in [73]). Cells have an antioxidant defense mechanism to counter the deleterious effects of ROS. For example, superoxide dismutase (SOD) is an antioxidant enzyme that converts superoxide free radical anion (O_2^-) into H_2O_2 and molecular oxygen (O_2) [75]. H_2O_2 must then be converted into H_2O by two other antioxidant enzymes: peroxisomal catalases and the family of glutathione peroxidases (GPx). In the event that H_2O_2 is not converted, then it may split into the hydroxyl radical (OH^\bullet), which can be dangerous because it can react with almost any macromolecule within a short diffusion distance. Cells, through the activity of nitric oxide synthase, are able to produce the free radical nitric oxide (NO). NO itself is hardly toxic and is in fact important in regulating various cellular functions. In fact NO has been suggested to increase aqueous humor outflow by relaxing the ciliary smooth muscles [76,77]. However, NO becomes dangerous when it spontaneously reacts with superoxide O_2^- , forming the powerful oxidant peroxynitrite ($ONOO^-$) [78]. Peroxynitrite is highly reactive and can damage biological molecules resulting in cell death (reviewed in [79]). In this way, the antioxidant defense mechanism also functions in minimizing the deleterious effects of reactive nitrogen species (RNS).

Chronic oxidative stress is recognized to be a major contributor to the aging process and various diseases including neurodegenerative diseases such as Parkinson's [80,81] and Alzheimer [82-84], cancer [72,85], and cardiovascular diseases [86]. Since POAG is an age-related disease, chronic oxidative stress is also suggested to have a role in the pathophysiology of this disease (reviewed in [87]). In POAG, both the RGCs and the anterior segment structures such as the TM are exposed to chronic oxidative stress conditions. TM cells are exposed to acute oxidative stress under normal physiological conditions [67]. The presence of cellular defense mechanisms in TM cells enables TM cells to quickly and effectively respond and adapt to their environment (Figure 2). Two cellular defense mechanisms present in TM cells are the

antioxidant system, which defends against ROS, and the proteolytic system, which removes unwanted biomaterials from the cell, many of which are products of oxidative stress-related damage. However, as aging progresses, the normal cellular defense mechanisms become less effective and the cell is less able to remove potential toxic materials such as ROS and misfolded proteins (Figure 3). The gradual accumulation of toxic materials will lead to an environment where the cells are exposed to chronic oxidative stress. We hypothesize that the cellular defense mechanisms, already compromised due to the aging process, become completely overwhelmed under such chronic oxidative stress conditions. Cell death will occur when the cells are no longer able to adapt to the environment. Since accumulation of ROS occurs with age, the loss of TM cells during the aging process may also be in part due to exposure of TM cells to chronic oxidative stress conditions. The presence of fewer TM cells as aging progresses could also be detrimental to the TM tissue as there are fewer cells to protect against ROS in the aqueous humor. Although it remains a likely possibility, evidence that chronic oxidative stress directly contributes to the loss of TM cells with age is currently lacking.

In comparison to non-glaucomatous individuals, the TM cells of glaucoma patients appear to have more oxidative stress-related damages, including the accumulation of oxidatively damaged DNA[88,89], proteins[90], and organelles, as well as lipid peroxidation products [91,92] (Figure 5). Oxidative stress can damage DNA, resulting in the formation of 8-hy-

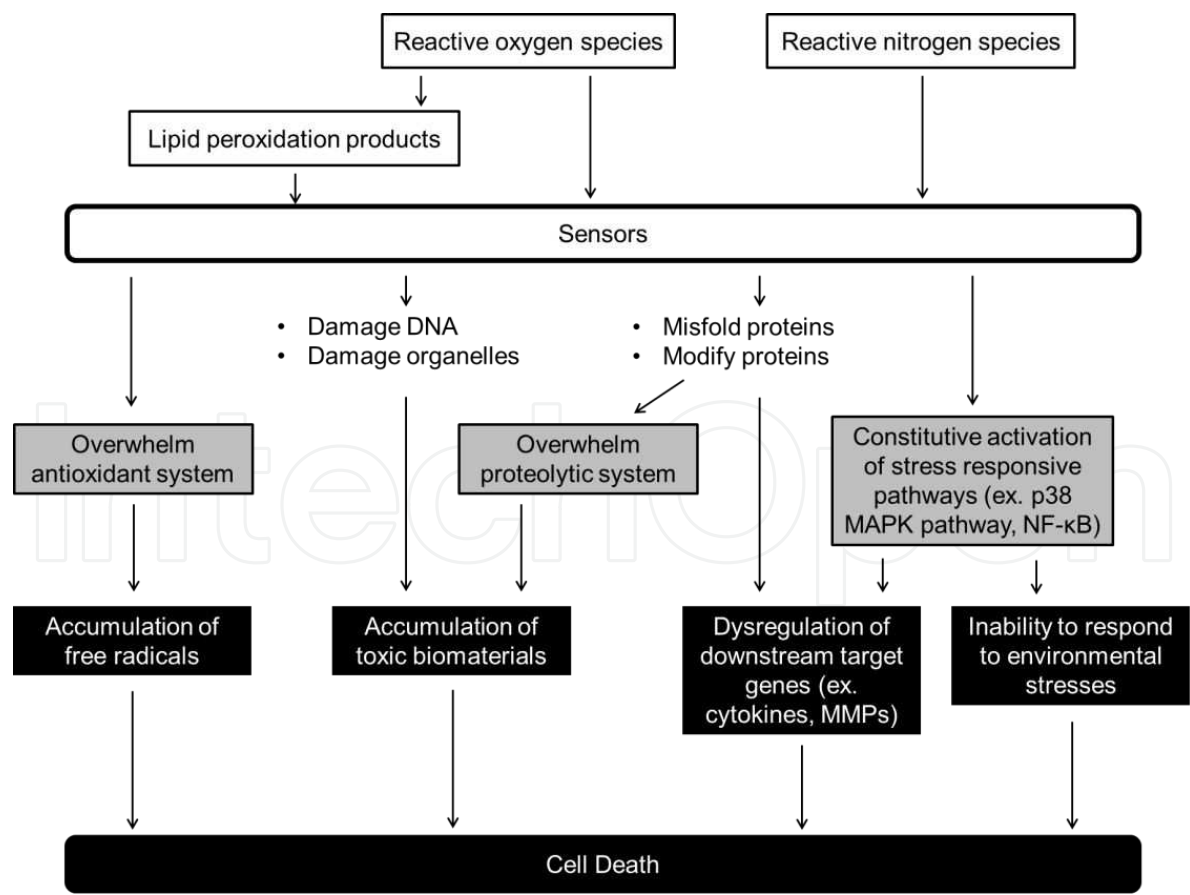


Figure 5. Overview of various oxidative stress-related effects on the cell, which would contribute to cell death.

droxy-2'-deoxyguanosine (8-OHdG). The levels of 8-OHdG were increased in DNA extracts from glaucomatous TM cells compared with healthy controls [88,89]. Also, aqueous humor and serum levels of 8-OHdG were significantly higher in glaucoma patients (n=28) compared with the age-matched control group of senile cataract patients (n=27) [93].

Despite having more oxidative stress-related damages, TM cells of glaucoma patients appear to have increased activity of some components of the antioxidant defense mechanism. Increased levels of glutathione peroxidase (GPx) and superoxide dismutase (SOD) activities were measured in aqueous humor of glaucomatous patients compared with the control group of senile cataract patients [94,95]. However, no apparent change in catalase activity levels have been detected [94,95]. Thus, at least some components of the antioxidant defense mechanism are functioning to prevent TM cell death under glaucomatous conditions.

Aqueous humor is both a source of free radicals and a source of antioxidants. Since low concentrations of free radicals are necessary for normal cellular function, TM cells rely on the very high content of antioxidants in the aqueous humor to achieve a balance that maximizes cell survival. High aqueous humor concentration of the antioxidant ascorbic acid (aka Vitamin C), which is about 20 times higher than in plasma [70], suggests that this antioxidant may be a major protector against free radicals in the eye [96-98]. Ascorbic acid has also been suggested to protect cells against ultraviolet light [98,99]. In addition to being an antioxidant, ascorbic acid is suggested to also have a role regulating the ECM of the TM. TM cells synthesize many types of glycosaminoglycans (GAGs) into the ECM including hyaluronic acid [100]. Ascorbic acid can increase hyaluronic acid synthesis [100]. Since hyaluronic acid has been shown to increase the expression of several MMPs [101], altered levels of this GAG would affect ECM turnover. Interestingly, Knepper *et al.* has shown that there is significantly less hyaluronic acid in the TM of POAG patients compared with the TM of normal subjects [102,103]. Thus, ascorbic acid is predicted to affect the aqueous outflow pathway by acting as an antioxidant and by regulating the ECM components that are important in maintaining the aqueous humor outflow pathway. Although some groups observed no difference in aqueous ascorbic acid levels between POAG patients and senile cataract patients [104,105], Lee *et al.* observed greater levels of ascorbic acid in the aqueous humor of POAG patients [106]. The difference in observation may be due to the great individual variation in ascorbic acid levels [105]. Nevertheless, ascorbic acid appears to play a protective role for TM cells.

In addition to the antioxidant system, the proteolytic system is another cellular defense mechanism present in TM cells. The proteolytic system is essential for the removal of oxidatively damaged proteins and organelles. The 20S proteasome, 26S proteasome, and immunoproteasome are the main cellular systems in eukaryotic cells that eliminate damaged proteins. The 20S proteasome tends to degrade oxidized proteins while the 26S proteasome degrades ubiquitinated proteins. In many tissues, including the TM, there is a decline in proteasomal activity with age. Caballero *et al.* reported that primary cultures of human trabecular meshwork (HTM) cells from healthy older donors (ages 66, 70, and 73) had decreased proteasomal activity compared with healthy young donors (ages 9, 14, and 25) [90]. Since the overall proteasomal content did not change between the older and younger donors, the decrease in proteasomal activity is most likely due to oxidation of the proteasomal subunits and the

overload of the proteasomal machinery with damaged proteins. Caballero *et al.* observed an increase in oxidized proteins in the older donors [90]. Accumulation of oxidized protein is not the only biomolecule detrimental to proteasomal function.

The accumulation of lipid peroxidation products in the TM is suggested to also contribute to proteasomal dysfunction [107]. Lipid peroxidation occurs when a ROS attacks a polyunsaturated fatty acid, thus initiating the lipid peroxidation chain reaction, which results in highly reactive aldehydes [108,109]. Lipid peroxidation products interact with protein, which results in modification to the protein structure and activity [109]. Accumulation of lipid peroxidation end products have been observed in many neurodegenerative diseases including Alzheimer's disease [110] and Parkinson's disease [111]. In glaucoma, an increase in lipid peroxidation end products, including diene and triene conjugates, and Schiff's bases, were observed in glaucomatous TM tissue (n=17) and aqueous humor (n=16) compared to age-matched controls (n=13 and n=17, respectively) [91]. In addition, Fernandez-Durango *et al.* measured increased levels of the lipid peroxidation mediator, malondialdehyde (MDA), in the aqueous humor of patients with terminal cases of POAG (n=38) compared to the cataract control group (n=48) [92]. Accumulation of lipid peroxidation products is predicted to have severe consequences on the TM by modifying proteins such as calpain-1. The calpains are a family of calcium-activated non-lysosomal cysteine proteases. In glaucomatous TM tissue, aggregated and degraded calpain-1 is present, but calpain-1 activity is lower compared with normal TM tissue [107]. In the TM of glaucomatous eyes, the lipid peroxidation products isolevuglandins, specifically iso[4]levuglandin E2, modifies calpain-1, thereby inhibiting calpain-1 activity. Although the physiological function of calpain-1 in the TM remains to be elucidated, calpain-1 modified by isolevuglandins is more prone to form larger aggregates. One of the major consequences of this modification is a disruption in the proteasomal machinery. This type of malfunction of the proteasomal machinery appears to be specific to the TM and does not occur in the posterior segment of the eye. Thus, accumulation of oxidative stress-related biomolecules along with a decrease in proteasomal activity with age perpetuates a vicious cycle that is postulated to greatly hinder cell survival.

7. Global change in gene expression in response to stress

As reviewed in the previous sections, cells of the TM are exposed to a variety of environmental stresses. The stresses can vary in form (mechanical, phagocytic, and oxidative), magnitude, and duration (acute or chronic). The antioxidant system and the proteolytic system are effective cellular defense mechanisms that protect cells. Recent advances in technology have shown that a change in the global gene expression profile is another major part of a cell's adaptive response to stress (reviewed in [112]). The change in gene expression profile in response to stress has revealed that signal transduction pathways are a necessary means of integrating complex signals and propagating these signals to effectors. In the next section, we will examine the specific sensors and signal transduction pathways that result in an appropriate response to stress in TM cells.

8. Sensors of TM cells

Cells have stress sensors that are highly specialized for survival in a particular environment. The specific mechanism of how TM cells sense various stimuli is largely unknown [48]. Mechanosensitive ion channels, specifically calcium-dependent maxi-K⁺ channels, are present in TM cells [113]. Stretch-activated channels located on the TM cell membrane are predicted to increase intracellular calcium levels. Another potential mechanism through which TM cells sense mechanical stress is the ECM. ECM receptors such as integrins are connected to the cytoskeleton, which is attached to the nuclear membrane. Thus, signals may be propagated from the extracellular environment where the mechanical stress occurs to the nucleus where gene expression can be altered in response to the stress [114]. Although the consequences of oxidative stress-related damages have been extensively studied, how the cell initially senses oxidative stress remains largely unknown [48,115]. In fact, the identification of oxidative stress sensors in any cell type has proven to be very difficult. In the future, identifying more sensors in TM cells will give insight into how TM cells achieve specificity in responding to specific stresses such as mechanical and oxidative stresses.

9. Global change in gene expression in response to mechanical stress

Exposure of TM cells to acute mechanical stress requires a quick and specific adaptive response to ensure maximal survival occurs. Recent studies examining the change in the global gene expression profile of TM cells has given insight into how TM cells are able to adapt and respond to the constant exposure to mechanical stress.

Several groups have examined the change in global gene expression profile of TM cells in response to mechanical stress [116-119]. Vittitow *et al.* and Vittal *et al.* both observed a change in expression of a large number of genes in response to mechanical stress. In TM from postmortem human donors, application of mechanical stress resulted in the upregulation of 40 genes and the downregulation of 14 genes [116]. Mechanical stretching of cultured porcine TM cells resulted in the upregulation of 126 genes and downregulation of 29 genes [117]. However, there was very little overlap in genes between the studies most likely due to the use of different experimental models as well of stochastic factors. Nevertheless, these studies reveal that TM cells appear to respond specifically to the *type* of stress. A large number of genes that changed expression levels were involved in ECM and cytoskeletal function, which is predicted to function in response to mechanical-stretch related changes to the cell and extracellular environment [117,118]. Several studies have shown that exposure of TM cells to mechanical stress results in increased levels of active MMPs, specifically MMP2 [120-123]. The MMP family of zinc proteases initiate ECM turnover, which has been predicted to regulate aqueous humor outflow facility by altering resistance. Furthermore, temporal variation of mechanical stretching resulted in a different gene expression profile indicating that TM cells are also able to respond specifically to the *magnitude* of mechanical stress.

Induction of stress response is thought to result in conditions that are detrimental to cell growth due in part to activation of cell cycle checkpoints [115,124]. Also, during stress response, the

cell diverts energy to the adaptive response and as a result, less energy is available for cell growth-related functions [112,125]. Thus, there is a continuous balance between cell growth and stress response. In order to achieve this balance, stress responses must be transient and be temporally restricted. Consistent with this theory, exposure to mechanical stress resulted in a change in expression of a large number of stress-related genes while few growth-related genes were affected [117]. Another related characteristic of stress response is the highly reversible nature of the global change in gene expression. After removal of stress, inactivation of the stress-induced signal transduction pathway occurs, likely because constitutive activation of stress response would be detrimental to the overall health of the cell. Although the specific reversal of the gene expression profile in TM cells after the removal of mechanical stress has not been examined, TM cells appear to physiologically return to the pre-stress state after a period of time. Perfusion of anterior segment cultures is an effective experimental model for examining TM function [126]. In this model, anterior segment explants are perfused with culture medium at a constant pressure, resulting in a stable and physiologically relevant flow rate [122,127]. Using perfused human anterior segment culture, Bradley *et al.* observed that doubling the flow rate resulted in immediate doubling of IOP [122]. However, after two days, TM cells lowered outflow resistance and thus, restored the IOP to pre-stress levels even under conditions where the flow rate remained doubled. In this study, the TM cells appear to reach a new homeostatic condition even when the stress is not removed.

Cells use multiple signal transduction pathways to integrate various input signals and coordinate an appropriate stress response. In TM cells, activation of signal transduction pathways appears to be important in mediating an appropriate stress response. Vittitow *et al.* observed that nearly 32% of genes altered in the global gene expression profile in response to mechanical stretch of TM cells functioned in various signal transduction pathways [116]. One particularly interesting signal transduction pathway is the stress-activated protein kinase (SAPK) pathways, a highly conserved signalling pathway in eukaryotes that are activated by many different environmental stresses. A rapid response to stress is essential to maximize cell survival. Thus, the SAPK pathway enables rapid phosphorylation of components of various signal transduction pathway so that a response occurs within minutes of initial exposure to the stress [128,129]. In mammals, the SAPKs are the p38 mitogen-activated protein kinases (MAPKs). There is evidence that the MAPK signal transduction pathway is involved in the TM cell response to mechanical stress [130]. In TM cells, the p38 MAPK pathway is suggested to modulate the regulation of stretch-induced cytokines such as TGF- β 1 and IL-6 [131]. Thus, the p38 MAPK pathway functions in co-ordinating and regulating signal transduction pathways in response to stress. However, in primary glaucomatous TM cells, Zhang *et al.* demonstrated that the p38 MAPK pathway is unresponsive to exogenous manipulation, including the administration of Interleukin 1 (IL1), which has been shown to activate the p38 MAPK pathway in non-glaucomatous TM cells [132]. Although examination of the p38 MAPK pathway *in vivo* is required, this pathway may be unresponsive in glaucomatous TM cells because it is already maximally activated [132]. The cause of this constitutive activation remains unknown. Nevertheless, the constitutive activation of the stress-responsive MAPK pathway is predicted to have serious consequen-

ces as the TM cells will lose its ability to mediate the stress response through the stress-dependent activation of the p38 MAPK pathway.

10. Global change in gene expression in response to oxidative stress

Despite the evidence that TM cells are exposed to oxidative stress, not much is currently known about the change in global gene expression profile in TM cells in response to chronic oxidative stress. Examining the effects of chronic oxidative stress on TM cells is especially challenging because it is difficult to experimentally create an environment where TM cells are exposed to chronic oxidative stress.

Porter *et al.* examined the global gene expression profile of phagocytically challenged TM cells under normal and acute oxidative stress conditions [133]. As avid phagocytes, TM cells are predicted to keep the aqueous humor outflow pathway clear of debris [55]. When TM cells were phagocytically challenged to *E. coli* under normal conditions, 1190 genes were upregulated and 728 genes were downregulated [133]. When TM cells were phagocytically challenged to *E. coli* under oxidatively stressed conditions at 40% O₂, 976 genes were upregulated and 383 genes were downregulated. Although many of the altered genes were involved in immune response, cell adhesion, and regulation of ECM, there were only 6 genes that were altered in both the normal and oxidatively stressed conditions. TM cells therefore appear to have distinct gene expression profiles specific to the type of stress. Under experimental conditions, different types of stresses tend to be examined one at a time to elucidate the response of the cells to that particular stress. However, TM cells under physiological conditions are simultaneously exposed to different stresses. Studies in yeast have shown that cells are able to combine and integrate these different signals and produce an adaptive response [128]. Thus, analyzing a combination of stresses in TM cells will possibly reveal the role that cross-protection plays in these cells. Cross-protection refers to the ability of cells to become resistant to stress after first being exposed to a sub-lethal stress [134].

Transcription factors are essential regulators of signal transduction pathway components. NF- κ B was identified as the transcription factor responsible for activation of many of the genes in the gene expression profile of phagocytically challenged TM cells including MMP1 and MMP3 [133]. The NF- κ B transcription factor has also been shown to mediate the activation of endothelial leukocyte adhesion molecule (ELAM1) and the inflammatory cytokine IL1 [135]. ELAM1 is a cell adhesion molecule that is readily expressed in glaucomatous TM cells [135-137]. Activation of ELAM1 and IL1 by the NF- κ B transcription factor in response to oxidative stress promotes cell survival. However, constitutive activation of NF- κ B is predicted to be detrimental to cell survival and even contribute to the development of glaucoma [135]. The NF- κ B transcription factor regulates the expression of numerous downstream target genes with varying functions including MMPs that regulate ECM turnover. Dysregulation of these downstream target genes is predicted to cause disruptions to TM cell function. In many situations, the altered gene expression returns to a steady-state level that is comparable to unstressed conditions even when the cells remain exposed

to a particular stress [125]. As mentioned previously, activation of stress-related genes during a stress response diverts energy and resources from cell growth. Thus, situations where steady state levels are not achieved can pose a great risk to the overall health of the cell, ultimately affecting its ability to survive.

11. Unspecific gene expression response

Studying the change in the global gene expression profile of TM cells reveals that a large number of genes are either upregulated or downregulated in response to various environmental stresses. Many of the genes that have altered expression do not appear to have any relevant function in the adaptive response to the specific stress. Analysis of global gene expression profiles in other systems such as *S. cerevisiae* yield similar findings of an unspecific gene expression response [138-140]. Furthermore, studies in *E. coli* have revealed that when cells are exposed to an unknown stress that the cell would not encounter under normal biological conditions, an unspecific and stochastic gene expression response was triggered [141,142]. Since cells may not have specific sensors to detect multiple stresses simultaneously, the unspecific stress response has been suggested to protect cells under multiple stress conditions by changing the expression of a large number of genes, some of which function in promoting a general adaptive response [125]. Furthermore, this unspecific and stochastic gene expression response may be an important evolutionary mechanism, thereby allowing cells to adapt to an unpredictable challenge [125]. Even though cells of the TM are in a dynamic environment with a multitude of challenges, the unspecific gene expression response may enable these cells to quickly and effectively adapt to a new steady state. In the future, distinguishing between stress-essential genes (necessary for immediate response) and stress-induced genes (most likely necessary for unspecific or long-term response) in TM cells is critical in understanding how TM cells adapt to stress in the long-term and prepare for subsequent stresses. Finally, although examining a particular stress-induced gene is important in elucidating its role in aqueous humor regulation, examining the *network* of genes altered in response to stress will provide further insight into the complex nature of the adaptive response of TM cells.

12. Effect of environmental stress on glaucoma-associated genes

Exposure of anterior segment structures, specifically the TM, to environmental stresses disrupts the aqueous humor outflow pathway and contributes to the development of glaucoma. Glaucoma, however, has a complex etiology. In addition to the environmental stress factors, genetic factors contribute to the development of this disease. At least 14 chromosomal loci have been identified for POAG (GLC1A to GLC1N) [143]. Currently three genes from these loci have been associated with glaucoma: *myocilin* (MYOC), *optineurin* (OPTN), and *WD repeat domain 36* (WDR36). Mutations in these three genes account for less than 5% of POAG cases [144]. Glaucoma is also a major consequence for many anterior segment dysgenesis disorders including Axenfeld-Rieger Syndrome (ARS) and Peter's anomaly. Mutations in the transcrip-

tion factor genes, *FOXC1* and *PITX2*, are associated with ARS [145-147]. How mutations in *FOXC1* and *PITX2* cause disease is not well understood. Recent findings have suggested that patients with these types of mutations may be more sensitive to environmental stresses [148,149]. The cells of the TM may be less tolerant when exposed to various stresses, resulting in dysregulation of aqueous humor outflow. In this section, we will take a closer look at the effects of environmental stresses on two genes, *MYOC*, which is associated with POAG, and *FOXC1*, which is associated with ARS.

MYOC was the first POAG gene to be reported [150-152]. Patients with *MYOC* mutations tend to present with juvenile and early adult-onset forms of POAG. However, the most commonly reported *MYOC* mutation, Q368X, is associated with later adult-onset POAG. *MYOC* is expressed in most ocular tissues [153] including the TM and is secreted into the aqueous humor [154,155]. The release of *MYOC* is associated with the release of exosomes. Signaling molecules within these exosomes is predicted to function in maintaining TM homeostasis [156]. Specific *MYOC* mutations appear to sensitize cells to oxidative stress. Joe *et al.* observed that Human Embryonic Kidney 293 (HEK293) cells stably transfected with the Y437H *MYOC* mutation have decreased expression of antioxidant genes and produced more ROS [156,157]. Also, more H₂O₂-induced cell death occurred in HEK293 cells overexpressing various *MYOC* mutations compared with wild type. The extent of cell death differed between mutants. Furthermore, 18 month old Y437H mutant mice had increased expression of ER stress markers and decreased levels of antioxidant proteins [157]. These findings suggest that patients with *MYOC* mutations are more sensitive to oxidative stress. The decreased ability to response to oxidative stress may contribute to the development of glaucoma earlier on in these patients' lives.

Anterior segment dysgenesis covers a wide spectrum of developmental anomalies that can affect the iris, cornea, lens, TM, and Schlemm's canal. We have already discussed the importance of the TM and Schlemm's canal in maintaining the aqueous humor outflow pathway. Disruptions in this pathway may result in increased IOP, which is a major risk factor of developing glaucoma. Glaucoma is estimated to develop in approximately 50% of patients with anterior segment dysgenesis [158-160]. Although the mechanism that leads to glaucoma may vary between different anterior segment dysgenesis disorders and even between individuals with the same disorder, recent findings suggest that environmental stresses affect the normal functioning of the disease-associated gene. Patients with ARS can present with a variety of ocular anomalies and systemic anomalies. Ocular anomalies include iris hypoplasia, corectopia, polycoria, and posterior embryotoxon while systemic anomalies include dental anomalies and redundant periumbilical skin. ARS patients with *FOXC1* mutations have a 50-80% risk of developing earlier-onset glaucoma [161]. As a transcription factor, *FOXC1* regulates the expression of a myriad of genes including genes that function in proteolysis, cell matrix adhesion, apoptosis, signal transduction, and stress response [148]. Berry *et al.* observed that *FOXC1* plays a role in TM cell viability by directly regulating the transcription factor FOXO1A which is involved in the cellular stress response pathway and apoptosis. Decreasing the expression of *FOXC1* increased the sensitivity of TM cells to oxidative stress. Tight regulation of the *FOXC1* transcription factor is essential because both a high (*FOXC1* duplications) and low *FOXC1* (loss of function mutations) gene dose results in anterior segment

dysgenesis phenotypes associated with glaucoma. Interestingly, FOXC1 itself appears to be responsive to stress as well (Y.A.I. and M.A.W. personal observations). Thus, the FOXC1 transcription factor appears to play an important role in responding to environmental stresses. Disruptions to normal FOXC1 function are predicted to disrupt the regulation of downstream target genes that are involved in executing a rapid and effective adaptive response. Therefore, ARS patients with FOXC1 mutations may have a compromised ability to respond to environmental stresses resulting in the early age of development of glaucoma. Thus, even in the case of anterior segment developmental disorders, oxidative stress appears to have an impact on the TM. Studying genes that are associated with both the primary and secondary glaucomas provide an invaluable tool to understanding the contribution of environmental stresses on the development of glaucoma.

13. Conclusion

The functional nature of the TM inevitably results in exposure of this tissue to a highly dynamic environment. Examining the functional roles of single genes have provided invaluable insight into how specific genes contribute to normal TM cell function and how these TM cells are able to respond to specific stresses. However, the recent analyses of global gene expression profiles have indicated that an extensive number of genes are involved in mediating the TM cell stress response. We are beginning to piece together how these single genes function as part of a 'network' of genes. Individual components of this network of genes are potential therapeutic targets for promoting cell survival and maintaining TM cell function. However, future research needs to examine how these genes interact with each other and the environment in a more physiologically relevant context; as part of a TM stress-response network. Understanding the network of genes that are involved in executing the adaptive response is complicated, but essential to developing effective treatments for anterior segment malformations and glaucoma.

Acknowledgements

We would like to thank Dr. Fred Berry and Mr. Tim Footz for critically reviewing this manuscript. Y.A.I. is supported by the Sir Frederick Banting and Dr. Charles Best Canada Graduate Scholarship provided by the Canadian Institutes of Health Research.

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