Elevated Intraocular Pressure induces Ultrastructural Changes in the Trabecular Meshwork

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**Abstract**

The elevation of intraocular pressure (IOP) can be caused by the obstruction of flow in the trabecular meshwork and the age of the individuals has been pointed as one risk factor influencing in developing glaucoma. This study was designed to elucidate the morphological and ultrastructural changes in the trabecular meshwork of young adult Göttingen minipigs eyes after experimentally inducing a moderate chronic elevation of intraocular pressure lasting for over 14 months. The method used was cautery of episcleral veins, located post trabecular in the flow pathway and thus not affecting the cells located in the trabecular meshwork. The tissue was analysed using electron microscopy in control and experimental eyes. An increase in the amount of fibrillar material in the subendothelial region with a decreased optically empty spaces and an increase in rough endoplasmic reticulum (rER) were observed in the young experimental eyes. By experimentally increasing the post trabecular resistance to the aqueous outflow, the present study showed that IOP elevation led to ultrastructural changes and thus concluded that changes in the trabecular meshwork can take place not only due to the advanced age, but by mechanical action on the cells as well.

**Keywords:** Glaucoma; IOP; Minipig; Trabecular meshwork; Schlemm canal; Ultrastructure; RER; Eye; Pore; Transcellular

**Introduction**

Elevated intraocular pressure (IOP) has been considered as one of the most important risk factors for primary open angle glaucoma (POAG). A causal role of elevated IOP in glaucomatous damage is supported by experimental studies on animals developed optic nerve cupping [1,2]. Clinical examples of elevated IOP are acute angle closure and replace by asymmetrical cupping and visual field loss. Risk of developing glaucoma is consistently greater with progressive high IOP. Other factors influencing glaucoma include age, ethnic group, genetic background, myopia, diabetes, migraine and vasospasm [3].

Aqueous humor passes through a porous connective tissue, the trabecular meshwork (TM), situated at the angle of the anterior chamber of the eye, near the base of the cornea and close to the ciliary body. From this angle aqueous flows into the Schlemm’s canal. The juxtacanalicular connective tissue (JCT) or cribiform meshwork is the region of the TM adjacent to the Schlemm’s canal. IOP is maintained normally through constant balance in the outflow resistant. A dramatic increase in outflow resistance has been implicated in the elevation of IOP leading to glaucomatous pathology. However the site of resistance has been contentious. The predominant sites have been juxtacanicular region of the trabecular meshwork and the endothelial cells of the inner wall of Schlemm's canal. JCT lies close to the inner wall of the endothelium of the Schlemm’s canal and is composed of loosely arranged extracellular matrix (ECM) into which JCT cells are embedded [4]. Abnormal regulation of aqueous flow through TM and JCT has been implicated in the elevation of IOP. Extra cellular matrix of JCT has been implicated as a barrier that may isolate the aqueous outflow [5].

In a series of papers, Lütjen-Drecoll, Rohen and others showed the structural changes of the outflow system with increasing age. Specifically, they found continuous loss of cells in the cribriform layer, the development of extracellular material and plaque formation. This sheath-derived plaque material in the JCT was postulated to lead to increased resistance outflow of aqueous humor and thereby the elevation of IOP that leads to glaucoma [5-8]. Lütjen-Drecoll [7] further concluded that chronic open angle glaucoma “cannot be considered simply an age dependent phenomenon” and the debate still persists as to the site of increased outflow resistance in POAG. Acott et al. [9] showed that sustained JCT stretching is the signal that initiates the IOP homeostasis response. It therefore is reasonable to assume that when unable to maintain a balanced outflow resistance, IOP would be elevated and may lead to glaucomatous conditions.

Tripathi [10] proposed a decrease in vacuolization of the inner wall of the endothelium of the Schlemm's canal in POAG. Other researchers found no difference in the number of giant vacuoles in normal and glaucomatous eye. However, in a recent study Overby et al. [11] showed that increased cytoskeletal stiffness of the Schlemm's canal endothelial cells lead to reduced pore formation by these cells.

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The porcine eye offers many features in common with the human eye [12-20]. The juxtacanalicular meshwork tissue of the aqueous humour outflow system in the pig has large similarities with the primate eye [21,22]. The JCT is located beneath the endothelium lining of the angular aqueous plexus, a thin zone that is morphologically analogous to the cribleform layer of primates.

Recently, Galdos et al. [23], studied in the Göttingen minipigs experimental glaucoma model the changes in the vasculature of the optic disk demonstrating that they were similar to those observed in human POAG. In previous studies Ruiz-Ederra et al. [24] demonstrated that moderated increase of IOP in pig eyes, caused by cautery of the episcleral veins, induced retinal ganglion cell death following a pattern similar to that described in humans. The pig model of glaucoma thus mimics cellular and physiological events that accompany the disease process in humans [25]. Moreover, Suarez and Vecino [26] have further demonstrated that as in human glaucomatous eyes, in a pig model of glaucoma, ELAM-1 is expressed within the TM endothelial cells. ELAM-1 is a transmembrane protein that transduces signals into endothelial cells, leading to the activation of a variety of signalling pathways, such as the mitogen-activated protein kinase (MAPK) signalling cascade. This model is thus further validated as a suitable tool for the study of human glaucoma and specifically to study the changes within the TM cells. The ELAM-1 expression on the outflow pathway at the anterior chamber level of human and pig glaucomatous eyes was proposed to be a response to the cellular stress generated by elevated intraocular pressure [26].

In the present study we analyzed the ultrastructural changes that take place in the JCT using the same animals that were analyzed for the pattern of the blood vessels and cupping of the optic disk [23]. The analysis was done by comparing the right eyes (control) versus the left eyes (experimental) that had undergone episcleral vein cautery.

Materials and Methods

There are numerous procedures to induce Glaucoma in animals. Pretrabecular and trabecular procedures will directly interfere with TM and are not suitable for our purposes. Laser induced scar formation will also directly interfere with the TM. Hypertonic saline solution injection into Schlemm’s canal will directly interfere with the endothelial cells of the SC. Hence, we used the episcleral venous cautery method to induce elevation of IOP in the present experiment.

The details of the method used to induce experimental glaucoma have been previously reported [19] and are summarized below.

Animals

This study was carried out according to the ARVO (The Association for Research in Vision and Ophthalmology) resolution on the use of animals in research and was approved by the university’s animal care and use committee. Eight Göttingen minipigs, four males and four females, were used in the present study. The average age of the animals at the beginning of the experimental period was five months (21.75 weeks, ranging from 19 to 24 weeks). At the end of the experiments the animals were approximately 19 months old. Minipigs become sexually mature at 3 to 4 month of age in male, weighing 7 to 9 kg, and 4 to 5 month of age in female, weighing 9 to 11 kg. All animals used in the present study were of the same age and were considered young adults. Animals were kept in the animal house under a 12:12 hour light: dark cycle, were fed twice a day and had ad libitum access to water.

Anaesthesia and analgesic procedure

The IOP was measured after administration of 1 drop of topical anaesthesia, (tetracain 0.1% and oxibuprocain 0.4%). The surgical interventions were carried out under general anaesthesia (10 ml/hour propofol given through an intravenous cannula inserted into the ear pinna) as well as topical anaesthesia (1 drop of local anaesthetic eye drops), with mechanical ventilation and monitoring of vital signs. After the operation and before the animal woke up, 1g of intravenous metamizole was injected as an analgesic treatment.

Procedure for the episcleral vein cautery (EVC)

The surgical procedure was carried out following a technique previously described for pigs by our group [19]. The operation was performed after 5 weeks of monitoring the IOP. Sham surgery was performed in the left eye in one animal where the eye underwent similar surgical procedure except for the cautery of episcleral veins. This eye was designated as the non-cauterized control. In the other seven animals, cauterization of three dorsal episcleral veins of the left eye was performed. The right eye served as the unoperated control. Three months after the first surgery a second operation was carried out on the previously operated eyes, with further cautery of two ventral episcleral veins to ensure continuation of elevation of IOP.

Monitoring of intraocular pressure

IOP was monitored in both eyes once a week using the TonoLab rebound tonometer (IcareTonomab, Finland). Measurements were taken at the same time of the day (11:00 am), after adding topical anaesthetic to the eye in awaken animals. Each recorded value was the mean of three to seven determinations. The animals were trained for the procedure. Overall, the IOP monitoring included a mean follow-up period of 14 months, with one weekly measurement.

Animal sacrifice and tissue extraction

At the end of the experiment, animals were sacrificed under general anaesthesia. Subsequently, eyes were enucleated and a section of the dorsal trabecular area, including the ciliary body and part of the cornea and retina, were taken from each enucleated eye. To maintain consistency, we chose only one location (12 o’clock position) in all animals for analysis presented here. All elements of the chamber angle that form the aqueous humor outflow system were obtained including the vessels of the angular aqueous plexus (equivalent to Schlemm’s canal in humans) and the cribriform or juxtacanalicular region, as well as the corneoscleral and uveal trabecular meshwork.

Preparation of samples

From the part of the chamber angle extracted, a sample containing cornea, sclera and iris was obtained in order to identify the vessels of the angular aqueous plexus and the corneoscleral trabecular meshwork. Subsequently, under higher magnification, we further cut three blocks of 1 mm³, per eye, of the trabecular meshwork. Trabecular meshwork specimens were fixed with 2.5% glutaraldehyde for 4 hours, rinsed in 0.1M phosphate buffer pH 7.4 and post-fixed with 4% OsO₄ for 2 hours. Tissues were then dehydrated in an ascending series of alcohol and embedded in Epon resin according to standard protocols.

Semi thin sections (1 μm) from each block were stained with toluidine blue. After capturing light microscopy images of the semi-thin sections, an area containing the vessels of the angular aqueous plexus and the juxtacanalicular or cribriform region was selected for further ultra-thin sectioning. Hence, from each experimental and control eyes at least 3 blocks were made. From each block, 3 semi thin sections and 6 ultrathin sections were obtained and analyzed.
Transmission electron microscopy

Ultra-thin sections of 70-90 nm were obtained using an ultramicrotome. Grid staining was performed with 2% uranyl acetate in distilled water and 2% lead citrate. The ultrastructural analysis was carried out using a Philips CM200 transmission electron microscope with a Gatan 696 TV camera. The vessels of the angular aqueous plexus between the corneoscleral region and the trabecular meshwork were located. Serial photographs of the sub endothelial region of the inner wall of the aqueous vessels were taken. To compare the control and the glaucomatous eyes, vessels of aqueous plexus were selected from the similar corneoscleral region, so that differences attributable to the location of the vessel were avoided.

Results

IOP measurements

Of the eight animals that underwent episcleral vein cauterization, 57.1% presented statistically significant differences (p<0.05) of IOP, when comparing the experimental left eyes (LE) and the control right eyes (RE), indicating that these animals had glaucoma [23]. IOP values of control eyes averaged 22.43 ± 4.46 mmHg and glaucomatous eyes averaged 26.04 ± 5.01 mmHg (p<0.01, Figure 1 and Table 1).

Control unoperated eyes

The aqueous vessels of the Göttingen minipig, which are located in the central region between the corneoscleral junction and the TM, were analysed using transmission electron microscopy (TEM). In the minipigs, as in humans, JCT is the outermost part of the cribriform region that lies in close proximity to the inner wall of the aqueous vessels and to the endothelium of the inner wall of Schlemm’s canal.

Control eyes

The subendothelial region was characterized by the existence of empty spaces, corresponding to the outflow pathway of the aqueous humour (Figure 2A). Thin scarce electron-dense fibrillar material that apparently connecting the subendothelial cells consider spacing (cribriform cells) to endothelial cells and also linked to clusters of electron dense elastic-like fiber was observed. In addition, few rough endoplasmic reticulum cisternae and optically empty spaces were observed (Figure 2A and 2C).

Experimental glaucomatous eyes

Animals with statistically significant chronic increases of IOP showed changes in the subendothelial region within the JCT where the empty spaces were less evident in the glaucomatous eyes compared to control eyes (Figure 2B). Analysis of the amount of plaque accumulation in the JCT showed that experimental eyes had smaller number of empty spaces, or the smaller size as seen in light microscopic section (Figure 2B), had more electron-dense material formed by elastic-like fibers forming bundles in occasions that fill the spaces between cells (Figure 2D) and the cells of this area had larger rER cisternae when compared to the controls.

Distinct changes in the fundus were noted in experimental animals when compared to controls. Figure 3 is a representative picture that shows that optic cupping and blood vessel patterns are dissimilar.

Discussion

It is believed that ageing is one risk factor for the development of POAG. It is further accepted that age related changes in the TM contribute to the development of elevated IOP, perhaps due to the impaired aqueous outflow resistance. Increased resistance to aqueous humor outflow, leading to the elevation of IOP, is a dominant risk factor for glaucoma [25,27-29]. The structures responsible for such an increase include JCT and SC [30-33]. In a recent paper Overby et al. [11] showed that endothelium of the Schlemm’s canal that lead to pore formation modulates the outflow resistance. In glaucomatous eyes there were reduced numbers of pores that led to the increase in the outflow resistance. These endothelial cells had more stiffness than control ones. Recent
Table 1: Intraocular pressure (IOP) values in control (Right Eye, RE) and glaucomatous eyes (Left Eye, LE).

<table>
<thead>
<tr>
<th>Animals Identity number</th>
<th>Mean IOP (mmHg) ± SEM</th>
<th>Mean IOP (mmHg) ± SEM</th>
<th>Statistical significance *p&lt;0.05</th>
</tr>
</thead>
<tbody>
<tr>
<td>control 00762</td>
<td>23.09 ± 5.39 control</td>
<td>25.64 ± 7.29 glaucoma</td>
<td>0.042</td>
</tr>
<tr>
<td>control 00763</td>
<td>23.28 ± 6.45 control</td>
<td>26.49 ± 6.92 glaucoma</td>
<td>0.015</td>
</tr>
<tr>
<td>control 00764</td>
<td>20.68 ± 5.13 control</td>
<td>25.20 ± 6.15 glaucoma</td>
<td>0.001</td>
</tr>
<tr>
<td>control 00765</td>
<td>23.61 ± 6.30 control</td>
<td>27.47 ± 7.54 glaucoma</td>
<td>0.005</td>
</tr>
</tbody>
</table>

Values are expressed as mean ± SEM. Results from statistical analysis are represented by *p<0.05, significant difference with respect to control RE (Wilcoxon signed-rank non-parametric test).

Figure 2: Control right eye (A, C,) and glaucomatous left eye (B, D) of the same animal. Representative figure including semithin (A and B) and ultrathin sections (C, D) corresponding to the same area. C and D shows a higher magnification of the aqueous vessels from A and B where the internal wall of the JCT are pointed by black arrows.. In C there are more optically empty spaces with less fibrillar material in the subendothelial region and less rough endoplasmic reticulum than in D. Asterisks indicate fibrillar material and arrowheads rER cisternae.

Figure 3: Eye fundus of minipig #7 (90713). The pictures were taken (A) before and (B) after 14 months of moderated elevation of intraocular pressure. Morphological changes could be observed, such as a paler appearance and increase in the cup-disc ratio. Optic disc excavation was identified by the curving of the retinal blood vessels at the disc margin.
other locations may show similar alterations in elevated IOP eyes. It is conceivable that in other quadrants of the eye, differences may exist and that the effects on ultrastructure may vary. However, the present results provide convincing evidence that elevated IOP produced changes at ultrastructural level by raising the extracellular matrix elements.

Our findings support observations on mechanosensing and signaling pathways in glaucoma in response to fluctuating shear stress such as intraocular pressure [34-36]. These findings suggest that the increase in IOP by itself can induce changes in the cribriform region. The increase in IOP seems to be a factor that mechanically perturbs the subendothelial cribriform and juxtacanalicular cells. This could lead to a secondary release of stress factors, inducing changes in the extracellular matrix that may result in an increase of the outflow resistance to the aqueous humor, a mechanism that has previously been suggested [37-44]. The observed changes in the juxtacanalicular connective tissue secondary to an increase in IOP may explain the progressive nature of this disease that is characteristic of elderly people. Although direct cellular measurements of JCT supporting its role in aqueous outflow resistance to the extracellular space by the subendothelial cribriform and juxtacanalicular cells. This could lead to secondary release of stress factors, inducing changes in the extracellular matrix that may result in an increase of the outflow resistance to the aqueous humor, a mechanism that has previously been suggested.

In conclusion, we propose that the modifications in the trabecular meshwork described in glaucoma are not only due to advanced age, initial elevation of IOP (experimentally induced in the present study) could induce changes in the trabecular cells which secrete extracellular matrix material, even in young individuals, thus initiating a vicious cycle that reinforces the chronic and progressive character of this disease.

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