Confocal microscopic examination of trabecular meshwork removed during ab externo trabeculectomy

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Aims: The aim of the ab externo trabeculectomy (AET) is to remove the external portion of the trabecular meshwork (ETM) responsible for the main aqueous outflow resistance in glaucoma patients, with no opening of the anterior chamber. ETM characteristics were evaluated with a confocal microscope.

Methods: A prospective comparative observational case series was performed in 60 consecutive medically treated patients with primary open angle glaucoma and eight postmortem normal donors' eyes that underwent AET. Once deroofting the Schlemm's canal (SC), a deeper dissection led to removal of a coherent membrane (ETM) which allowed satisfactory aqueous egress through the remaining intact internal trabecular meshwork (TM) layers. After fixation with acetone and immunostaining with anti-vimentin antibody, ETM were analysed with a confocal microscope.

Results: Glaucomatous ETM (mean thickness: 29.5 (7.6) μ m) were characterised by a severe paucicellularity compared with the controls (respectively 37.3 (9.7) cells/area and 167.5 (24.9) cells/area, $p < 10^{-4}$). ETM analysis showed involvement of both cribriform and corneoscleral layers. ETM cell density was significantly decreased in case of preoperative fluorometholone instillation.

Conclusion: Paucicellularity of glaucomatous TM is confirmed by this original technique. Structural characteristics of the ETM, whose removal allows satisfactory aqueous egress, suggest that aqueous outflow resistance not only involves inner wall of SC and juxtacanalicular meshwork but also corneoscleral trabecular layers.

Ab externo trabeculectomy (AET) is a new glaucoma filtering surgery that has been described in recent years and is used to improve the safety of filtering procedures. The main concept of this procedure is to selectively remove the portion of the trabecular meshwork (TM) involved in aqueous outflow resistance in patients with primary open angle glaucoma, while leaving intact the inner part of the TM.^{1–3} The integrity of inner trabecular membrane avoids a sudden peroperative decompression of the eye, which can be responsible for postoperative complications such as hypotony with choroidal detachment or flat anterior chamber, as has been reported after classic trabeculectomy. The success rate of this surgical procedure proved to be satisfactory^{4–9} and comparable to that of trabeculectomy, with fewer complications.^{10–11}

The sustained decrease in intraocular pressure (IOP) level after AET suggests that a significant part of the aqueous humour outflow resistance is removed. It was previously reported that the main portion of the TM involved in aqueous outflow resistance in glaucoma patients was the inner wall of Schlemm's canal (SC) and the adjacent trabecular layers—namely, the cribriform TM.^{12–13}

The present study aimed to evaluate, by confocal microscopy, the structural characteristics of this external part of the TM removed during AET, allowing us to obtain a satisfactory aqueous flow through the remaining part of the inner TM layers. Results were compared with those observed in fresh enucleated normal donors with no history of ophthalmic disease and removed under the same dissection procedure.

MATERIALS AND METHODS

Sixty eight external trabecular meshwork membranes (ETMs) were obtained from 60 consecutive primary open angle glaucoma patients and eight normal eyes. Glaucoma patients, six black and 54 white, aged 59.3 (SD 14.6) years (range 29–82 years), underwent AET by three different surgeons according

to the same surgical technique, from September 1999 to August 2000. Informed consent was obtained from all patients for analysing the specimen removed during the surgery they underwent. This study was conducted according to the tenets of Declaration of Helsinki.

The mean (SD) duration of glaucoma disease and treatment was 5.7 (7.1) years (range 6 months to 30 years) and the mean (SD) number of preoperative medical treatments was 2.3 (0.9) (range 1–4 drugs). Ten eyes had previously undergone an argon laser trabeculoplasty (1–15 years before surgery, mean 6.7 (SD 5.7) years), but none had undergone previous filtering surgery. All eyes had typical glaucomatous visual field defects as assessed by automated perimetry (early defects: eight cases, moderate defects: 17 cases, severe defects: 35 cases, according to Hodapp classification¹⁴). Indications for surgery were uncontrolled IOP despite medical treatment or progression of visual field defects and/or papillary excavation despite IOP less than 20 mm Hg. Thirty two patients had preoperative fluorometholone (Flucon, Alcon, Forth Worth, TX, USA) instillation (FI) 3 weeks before surgery. Fluorometholone instillation was systematically used preoperatively by one of the three surgeons, independently of the conjunctival status, in order to reduce postoperative conjunctival inflammation and according to a previous report by Broadway *et al.*¹⁵

All eyes underwent the same surgical procedure as previously described.⁶ Under a conjunctival fornix based flap, a first one third scleral thickness flap was dissected. In 12 eyes (six black patients, six white patients with preoperative IOP less than 15 mm Hg), a sponge soaked with mitomycin C (0.2 mg/ml) was applied for 3 minutes on the sclera before any

Abbreviations: AET, ab externo trabeculectomy; CM, confocal microscope; ETM, external trabecular membrane; FI, fluorometholone instillation; IOP, intraocular pressure; SC, Schlemm's canal; TEM, transmission electron microscopy; TM, trabecular meshwork

Figure 1 (A) Normal external trabecular membrane (ETM) showing three superimposed layers of tissues (CM, $\times 400$). Plane of the endothelial cells with red stained nuclei with no vimentin immunostaining (right side, arrowheads). Numerous juxtacanalicular cells (red stained nuclei with propidium iodide) characterised by star-shaped cytoplasmic expansions (green anti-vimentin immunostaining, arrows) and lying in a loose extracellular matrix. Plane of the corneoscleral meshwork with its yellow green fluorescent fibres (asterisks) located beneath the two previous planes. (B) Glaucomatous juxtacanalicular trabecular meshwork (CM, $\times 500$). The cellular density (red stained nuclei) is considerably reduced with juxtacanalicular cells showing markedly enlarged cytoplasmic expansions (green anti-vimentin immunostaining, arrows). Clump of few endothelial cells with no vimentin immunostaining (upper right, arrowheads). (C) High magnification of typical star-shaped cytoplasmic expansions (green anti-vimentin immunostaining, arrows) of glaucomatous juxtacanalicular cells (CM, $\times 800$). (D) Glaucomatous ETM (CM, $\times 800$). The focal plane is located at the frontier between the juxtacanalicular meshwork which is characterised by few star-shaped cells (bright green anti-vimentin immunostaining, arrows) and the corneoscleral trabecular layers whose extracellular matrix is organised in regular autofluorescent fibrillar bands (asterisks).

scleral dissection and rinsed with 150 ml of sterile saline 0.9%. A second scleral flap was dissected in the plane of SC. The external wall of SC was opened and removed. The bed of SC was carefully scraped in order to remove trabecular tissues so that satisfactory aqueous outflow could be observed through the remaining internal trabecular layers. When scraping the bed of SC, a constant cleavage plane in the trabecular layers allowed us to remove a homogeneous external trabecular membrane (ETM) leading the aqueous humour to egress through the thin remaining inner trabecular layers. In a small number of cases, the scraping of the bed of SC had to be done in different steps, with subsequent dissection of thinnest tissues, in order to obtain aqueous humour outflow. In these latter cases, the patients were excluded from the study. The integrity of the inner layers of the TM was checked with a sponge. All cases with evidence of microperforation of the innermost trabecular layers were not analysed. The removed

ETM was analysed using confocal microscopy as previously published in a preliminary study.¹⁶ These glaucomatous ETMs were compared with those obtained in eight enucleated post-mortem normal donors (mean age 67.3 (SD 7.8) years) in which the same surgical procedure was performed once the vitreous cavity had been filled up with sterile saline in order to increase the IOP up to 25 mm Hg.

Each ETM was laid down on a glass slide, fixed with acetone and immunostaining with vimentin was performed according to previously validated methods.¹⁷ Anti-vimentin antibody that binds mesenchymal cells was used in order to identify trabecular cells. Mouse monoclonal anti-vimentin IgG (Dako SA, Copenhagen, Denmark, clone Vim 3B4) was used in a dilution of 1:50 in phosphate buffered saline (PBS, pH: 7.4, Biomérieux, Lyons, France). The antibody was applied on the ETMs for 30 minutes at room temperature and then washed three times with PBS. The ETMs were then incubated in the

Figure 2 (A) Normal ETM in the plane of the corneoscleral trabecular layers (arrows), with numerous trabecular cells as assessed by red stained nuclei and yellow green autofluorescent fibres (CM, $\times 700$). (B) Glaucomatous corneoscleral trabecular layers of the ETM. The cellular density (red stained nuclei) is reduced compared with that of normal donors. The extracellular matrix is organised in regular green autofluorescent fibres (arrows, CM, $\times 700$). (C) Glaucomatous corneoscleral layers of the ETM with pigment (CM, $\times 550$).

dark for 30 minutes at room temperature with the secondary antibody in a dilution of 1:50 (goat anti-mouse immunoglobulins F(ab')₂, Dako SA). The ETMs were then rinsed twice with PBS, cell nuclei were stained with propidium iodide (Immunotech, Marseilles, France) in a 1:500 dilution in PBS, for 5 minutes, and then washed again with PBS. A drop of antifading medium (Vectashield H-1000, Vector Laboratories, Inc, Burlingame, CA, USA) was added and the ETMs were analysed with a confocal microscope (Nikon EZ 2000, equipped with epifluorescence microscope E800). The confocal microscope allowed to analyse the ETMs laying flat on the slide, from the top to the bottom, thanks to the confocal procedure that ensures only the light reflected from structures located in the same plane is analysed. In order to evaluate the ETM thickness, we used Z-scan analysis by steps of 1 μm sections at three different locations chosen at random throughout the specimen. Results were expressed as the mean values of these three measurements.

For each ETM, the cytoarchitectural characteristics were described and mean cellular density was calculated from five cell counts performed by counting red stained cell nuclei in five different sections (areas $50 \times 50 \mu\text{m}$, thickness 1 μm) randomly selected throughout the specimens.

ETM characteristics, thickness, and cellular density were also evaluated according to patient age, race, duration of

preoperative medical treatment, number of preoperative medications, previous trabeculoplasty, preoperative instillation of fluorometholone, and peroperative use of mitomycin. Statistical analyses were done by the Mann-Whitney and Z correlation tests with a significance at $p < 0.05$.

At the same time, five ETMs were cut in their middle in order to evaluate the ETM both with the confocal microscope and by transmission electron microscopy (TEM). For TEM, specimens were fixed by immersion in a mixture of 2.5% glutaraldehyde, washed and postfixed in osmium 1%. The tissue was dehydrated and embedded in Epoxy resin (Epon). Semi-thin sections 1 μm thick were stained with toluidine blue and observed. Ultrathin sections were prepared for TEM examination with the Philips CM 10 electron microscope.

RESULTS

The mean (SD) ETM thickness evaluated with the confocal microscope (CM) was not statistically different in glaucoma patients (29.5 (7.6) μm , range 12–50 μm) compared with normal donors (34.4 (10.4) μm , range 20–50 μm , not significant, NS). A monolayer composed of endothelial cells of the inner wall of SC could be seen in most samples. However, in glaucoma patients, these cells were not arranged in a confluent manner and mostly remained scattered throughout the specimen. The outermost portion of the TM close to the SC

Table 1 Mean (SD) external trabecular membrane (ETM) cell density and ETM thickness according to race, previous argon laser trabeculoplasty (ALT), preoperative use of fluorometholone (FI), and peroperative application of mitomycin (MMC)

	Mean (SD ETM thickness (μm))*	Mean relative ETM cell density† (area $50 \times 50 \mu\text{m}$)
Normal ETM (n=8)	34.4 (10.4)	167.5 (24.9)
Glaucoma patients (n=60)	29.5 (7.6)	37.3 (9.7)
Black patients (n=6)	34.3 (7.1)	25.5 (16.6)
White patients (n=54)	28.9 (7.1)	39.4 (30.4)
Previous ALT (n=10)	29.1 (5.9)	22.6 (16.1)
No previous ALT (n=50)	29.5 (7.6)	39.6 (30.7)
Preoperative FI (n=32)	30.8 (6.9)	26.3 (16.9)
No preoperative FI (n=28)	28.0 (7.4)	46.4 (34.6)‡
Peroperative MMC (n=12)	29.2 (7.6)	37.2 (28.8)
No peroperative MMC (n=48)	29.8 (7.5)	37.8 (32.2)

*Mean count of 3 sections in different areas of the ETM specimens.

†Mean count in 5 different areas and planes.

‡ $p=0.02$.

was characterised by an extracellular matrix with irregularly arranged fine fibres often appearing coiled or bent, so that empty spaces of various sizes were formed (Fig 1). In this portion of the TM, trabecular cells stained for anti-vimentin antibody were characterised by large cytoplasmic expansions as star-shaped cells (Fig 1B, C). Cytoplasmic expansions were markedly and consistently longer in glaucoma samples compared with normal ones (Fig 1B). The mean (SD) thickness of this outer portion of TM was $7.6 (2.5) \mu\text{m}$ (range $3\text{--}12 \mu\text{m}$) in glaucoma patients and $10.3 (3.4) \mu\text{m}$ in normal donors (NS). Beneath this outer part of TM, trabecular cells with no cytoplasmic expansions and poorly stained for anti-vimentin antibody were embedded in a regularly arranged fibrillar extracellular matrix, identified according to non-specific autofluorescence and strongly suggesting corneoscleral lamellae (Figs 1D, 2A).

In the whole ETM thickness, the confocal laser stimulation allowed us to visualise elastic fibres that appeared to be autofluorescent.

The main glaucomatous ETM characteristic evaluated with the confocal microscope was a significant reduction in the number of trabecular cells compared with the controls (respectively: $37.3 (9.7)$ cells/area $50 \times 50 \mu\text{m}$, range $5\text{--}150$; and $167.5 (24.9)$ cells/area $50 \times 50 \mu\text{m}$, range: $120\text{--}200$, $p<0.0001$). This rarefaction in cellular density characterised both the cribriform and the corneoscleral TM layers (Figs 1B–D, 2B). In most samples, pigment could also be visualised (Fig 2C).

In glaucoma patients, the mean ETM thickness was negatively correlated with patient age ($r = -0.32$, $p = 0.01$) and with duration of glaucomatous disease ($r = -0.32$, $p = 0.01$), meanwhile age and duration of glaucoma disease were positively correlated ($r = +0.34$, $p = 0.008$). Furthermore, there was no correlation between mean relative trabecular cell density and glaucoma patient age, duration, or severity of glaucoma disease and number of preoperative antiglaucoma medical treatments.

Mean (SD) age of glaucoma patients who were black was significantly lower compared with mean age of glaucoma patients who were white (respectively $47.1 (11.2)$ years and $60.9 (13.6)$ years, $p = 0.02$) but duration of their glaucoma disease was not statistically different. Mean ETM thickness was slightly higher in black patients compared with white patients (respectively $34.3 (7.1) \mu\text{m}$ and $28.9 (7.1) \mu\text{m}$), and mean trabecular cell density was slightly lower (respectively $25.5 (16.6)$ and $39.4 (30.4)$). However these differences did not reach significance.

In patients with and without previous trabeculoplasty, the mean (SD) ETM thickness was not statistically different (respectively $29.1 (5.9) \mu\text{m}$ and $29.5 (7.6) \mu\text{m}$, NS). The trabecular cell density was lower in patients with previous

trabeculoplasty compared with patients without previous trabeculoplasty (respectively $22.6 (16.1)$ and $39.6 (30.7)$); however, this difference did not reach significance ($p = 0.088$).

Mean (SD) trabecular cell density was significantly lower in patients with preoperative fluorometholone instillation (FI)

Figure 3 (A) Transverse sections of glaucomatous ETM with endothelial cells (arrow), juxtacanalicular meshwork (JC) containing few cells in a loose extracellular matrix, and a part of the corneoscleral meshwork composed of regularly arranged fibres (CS) (TEM, $\times 3000$). (B) Transverse sections of glaucomatous ETM: endothelial cell bumping in the lumen of Schlemm's canal (arrow), pigmented juxtacanalicular meshwork (JC) and few corneoscleral layers beneath (CS) (TEM, $\times 14\,000$).

compared with patients without any anti-inflammatory treatment (respectively 26.3 (16.9) and 46.4 (34.6), $p = 0.02$) while ETM thickness was not statistically different (respectively 30.8 (6.9) μm and 28.0 (7.4) μm , NS).

The use of peroperative mitomycin C had no influence upon the number of trabecular cells (37.2 (28.8) cells in the MMC group and 37.8 (32.2) cells in the group without MMC, NS) and no influence upon the ETM thickness (29.2 (7.6) μm in the MMC group and 29.8 (7.5) μm in the group without MMC, NS). In addition, the mean cell density evaluated in the subpopulation of patients without preoperative fluorometholone instillation was not statistically different between patients with (46.0 (38.8)) and without peroperative MMC use (43.4 (16.4)). The main results are reported in Table 1.

In order to eliminate the eventual confounding influence of trabeculoplasty and/or use of MMC in patients on cell count in patients with preoperative FI, statistical comparisons were also performed after excluding patients with previous trabeculoplasty and/or MMC use. The ETM cell density remained significantly decreased in the FI group, even when excluding patients with preoperative trabeculoplasty and those with peroperative use of MMC ($p = 0.02$, and $p = 0.01$, between FI and non-FI specimens, respectively).

The analysis of the ETM with the electron microscope showed that the ETM involved the endothelium of Schlemm's canal, the cribriform meshwork, and corneoscleral trabecular layers (Fig 3A and B).

DISCUSSION

The aim of non-penetrating filtering surgery is to create a filtration, responsible for an increase of outflow facility, with no opening of the anterior chamber^{1-11 18} since the inner layers of the trabecular meshwork are left intact.¹⁹

The most important point of ab externo trabeculectomy is the selective removal of the portion of the TM that is supposed to be responsible for the main aqueous outflow resistance. Although the exact location of aqueous outflow resistance is still controversial, several studies support the evidence that it is mainly located in the inner wall of SC and the adjacent trabecular layers.^{20 21} According to Mäepea and Bill¹² who used an in vivo micropuncture technique in monkeys to evaluate the pressure levels in different portions of trabecular meshwork under various intraocular pressure levels, 74% of aqueous humour outflow resistance is located in a 14 μm area adjacent to the SC—that is, in the cribriform trabecular meshwork.

It is known from histological studies that the cribriform TM is composed of star-shaped trabecular cells connected to each other and to the inner wall endothelium of SC and immersed in loose extracellular matrix. These cribriform cells are actively phagocytic and are involved in the turnover of the extracellular matrix which contains type I and type III collagen,²² chondroitin sulphate and dermatan sulphate glycosaminoglycans,²³ and a dense network of elastic-like fibres, connected to the inner wall of SC by connecting fibrils.²⁴ Anterior ciliary muscle tendons are anchored within this cribriform plexus so that contraction of the muscle may influence the width of the cribriform layer and change the form of the intercellular spaces of this area, thereby influencing outflow resistance.²⁵

Ab externo trabeculectomy is based on the assumption that the main outflow resistance in glaucomatous patients could probably be relieved by the removal of the external part of the TM (ETM), while the aqueous humour leaves the anterior chamber through the remaining intact trabeculodescemet membrane.¹⁹ However, to our knowledge, there is no available information concerning the composition of this ETM. Our study is the first one to report an ex vivo analysis of the ETM with a confocal microscope. This original method allows us to obtain optical horizontal sections through the entire length

and thickness of the removed TM layers by steps of 1 μm or less, while standard light or electron microscopy procedures allow analysis of TM samples from sagittal or transverse TM sections. In all our ETM samples, it was possible to visualise the trabecular cribriform meshwork that was identified through the characteristic presence of star-shaped cells strongly expressing vimentin. This outer part of the TM was located beneath the inner wall of SC that could be identified through the monolayer organisation of the endothelial cells lying on the same confocal plane. The mean thickness of the trabecular cribriform layers in our study was 7.6 (2.5) μm (range 3–12 μm). This finding is consistent with the thickness of the juxtacanalicular meshwork as was previously described using transmission electron microscopy.^{26 27}

Several modifications of the glaucoma cribriform TM have been described that can be involved in aqueous outflow resistance, although the main ultrastructural components thus responsible for the aqueous outflow resistance are still not completely known.^{28 29} In our glaucomatous samples, discontinuities between adjacent endothelial cells were common, as was previously reported in Grierson's study.³⁰ Although a loss of cells during the surgical removal of the ETM cannot be excluded, this finding could suggest an impairment of the endothelial wall of Schlemm's canal in glaucomatous patients, as previously shown at places where the cribriform extracellular matrix was deeply modified.²⁸

The main trabecular modifications described in glaucoma patients resemble age related trabecular meshwork changes,^{31 32} which suggests that the pathology of primary open angle glaucoma (POAG) in the outflow system is that of an exaggerated ageing process.³⁰ These changes involve modifications of the trabecular matrix with deposit of so called plaque material,²⁸ thickened trabeculae, atypical collagen and abundance of long spacing collagen²⁴ and a decrease in trabecular meshwork cellularity. Our study confirms the dramatic decrease in trabecular cellular density, both in the cribriform meshwork and in the corneoscleral trabecular layers, which constitutes the main modification of the POAG meshwork first described by Alvarado *et al*, especially in advanced glaucomatous disease.³² It has been suggested that excessive cell loss was an early pathological event in the outflow system in POAG.³³ Our results are consistent with this finding since the decrease in trabecular cell density in glaucoma patients was not correlated with the severity or the duration of glaucoma disease. However, our results should be interpreted with caution since most of our patients had moderate to severe glaucoma disease at the time of surgery, which might have prevented us from finding any correlation between cell loss and severity of glaucoma.

This trabecular cell depletion has been involved in trabecular structural alterations such as trabecular thickening, trabecular fusion, and accumulation of extracellular material in the endothelial meshwork.³² The decline in cellularity was previously found to be similar to but greater than that observed in advanced age meshwork, with the inner tissues of the trabecular meshwork affected, especially the corneoscleral trabecular meshwork.^{24 32} Our results are consistent with this finding since the loss of trabecular cells we reported in glaucoma samples involved both the cribriform and the corneoscleral trabecular layers. We did not find any correlation between trabecular cell density and glaucoma patient age, which suggests that glaucoma disease might be directly responsible for the trabecular cell decrease irrespective of cell loss related to age, as was previously reported by Alvarado.³⁴

There are several hypotheses concerning the decline in trabecular cellularity in glaucoma patients.^{30 35-39} Grierson hypothesised that there was a migration of trabecular cells from the trabeculae to the endothelial meshwork in early primary open angle glaucoma.³⁰ This hypothesis could explain the "activated" and enlarged trabecular cells in the endothelium meshwork that he reported in his study. Our results are

consistent with this hypothesis since we observed, in glaucoma samples, cribriform cells with larger cytoplasmic expansions compared to control samples.

Laser trabeculoplasty was shown to be associated with meshwork cell activation, migration, and fibrosis.⁴⁰ In our study, trabecular cell density was slightly lower in patients with previous trabeculoplasty compared with patients without, despite this difference did not reach significance ($p=0.088$). Since the filtering surgery was indicated in those eyes after failed argon laser trabeculoplasty, which has been performed a long time before—from 1 to 15 years—it can be supposed that argon laser therapy failed to stimulate trabecular cells enough and to prevent cell death resulting from glaucoma. Another explanation for the trabecular cell loss could be the cytotoxic effect of antiglaucoma drugs, which has been described in vitro on trabecular cell cultures.^{17,41,42} In this study, however, we did not find any correlation between the number of cells and the number of preoperative medications, which suggests that the local medical treatments have little influence on trabecular cell survival. Nevertheless, our study showed that patients who had preoperative fluorometholone instillation had statistically less trabecular cells compared with those without. As previously reported by Broadway *et al*,¹³ fluorometholone was prescribed in order to decrease the incidence of preoperative conjunctival inflammation and to reduce inflammatory cell infiltrates with the further aim of improving filtering surgery outcome. Even if a direct toxicity of fluorometholone on trabecular cells leading to cell death cannot be excluded, it could be most likely suggested that fluorometholone might reduce the number of inflammatory cells in the TM, thus explaining the difference in cell number between TM samples with and without preoperative fluorometholone instillation. The possible presence of inflammatory cells in the TM, as previously reported,¹⁷ could have interfered with trabecular cell count, thus preventing any conclusion concerning the potential responsibility of preoperative medical treatment regarding primary trabecular cell survival. Immunostaining with markers of immune cells and ultrastructural analysis of the ETM would be necessary to further differentiate subpopulations of cells in the TM. Thus, the responsibility of the topical glaucoma treatment in trabecular cellularity cannot be definitively excluded and will require further studies specifically aimed at this issue.

As it has been previously reported that the use of MMC applied on the sclera in glaucoma filtering surgery might be involved in postoperative chronic hypotony through direct ciliary toxicity,⁴³ the question of the toxicity of the mitomycin on trabecular cells has arisen. The trabecular cell density was similar in eyes with and without preoperative application of MMC in our study. Since nuclei of dead cells can be stained by propidium iodide, it was not possible to differentiate live from dead cells, so the question of direct MMC toxicity on trabecular cells remains unanswered by this technique.

In conclusion, with this original method using confocal microscopy, our study reported the structural characteristics of the external trabecular membrane removed during ab externo trabeculectomy and necessary to provide satisfactory aqueous humour outflow through the remaining intact part of the trabecular meshwork layers. This external trabecular membrane not only involved the trabecular cribriform layers but also a part of the corneoscleral layers, as confirmed with the TEM study. These results suggest that aqueous outflow resistance is not only located in the juxtacanalicular tissues. The decrease in cellular density was the most striking feature of the ETM in glaucomatous patients and appeared to be poorly influenced by local treatment of preoperative glaucoma. However, the even greater loss of trabecular cells in the case of preoperative local use of anti-inflammatory drops suggested the possible infiltration of the trabecular layers by inflammatory cells in glaucoma patients treated long term. The hypothesis of a potential trabecular inflammation

secondary to long term local glaucoma medication cannot be excluded. Further investigations are necessary to confirm or to invalidate this hypothesis.

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