Ex vivo excimer laser ablation of cornea guttata and ROCK inhibitor-aided endothelial recolonization of ablated central cornea

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ABSTRACT.

Purpose: To determine whether excimer laser ablation of guttata is a viable strategy for removal of diseased tissue in Fuchs’ endothelial corneal dystrophy (FECD) on excised human Descemet membranes and whether an excimer laser-created wound on healthy human corneas ex vivo is recolonized with corneal endothelial cells.

Methods: Descemet membranes of FECD patients and corneal endothelium of normal human corneas were ablated ex vivo using an excimer laser licensed for glaucoma surgery. Specimens were kept in cell culture medium supplemented with 10 μM of rho-kinase inhibitor ripasudil. Corneal endothelial cell regeneration was observed using light and electron scanning microscopy. Furthermore, the whole corneal samples were evaluated by haematoxylin/eosin staining and immunohistochemical analysis using antibodies against Na+/K+-ATPase.

Results: Gutttae and corneal endothelium could be ablated with an excimer laser without total ultrastructural damage to the Descemet membrane or stroma. Nearly complete endothelial wound closure was accomplished after 26–38 days in treated corneas. Light and electron scanning microscopy suggested the establishment of a layer of flat endothelial cells. Additionally, Na+/K+-ATPase expression could only be observed on the inner side of the Descemet membrane.

Conclusion: Our proof of concept study demonstrated that excimer lasers can be used to ablate diseased tissue from excised FECD Descemet membranes ex vivo. Additionally, corneal endothelial cells recolonize a previously ablated endothelial area in healthy human corneas ex vivo under treatment with ripasudil. Thus, our results are the first experimental basis to further investigate the feasibility of an excimer laser ablation as a graftless FECD treatment option.

Key words: corneal endothelium – corneal graft – Descemet membrane – endothelial keratoplasty – excimer laser – Fuchs’ endothelial corneal dystrophy

Introduction

Fuchs’ endothelial corneal dystrophy (FECD) is the most common dystrophy of the corneal endothelium leading to a significant decline in visual acuity. A gradual loss of endothelial cells leads to reduced corneal deturgescence via loss of Na+/K+-ATPase density (McCartney et al. 1987; McCartney et al. 1989) with subsequent corneal oedema (Elhalis et al. 2010) and opacity (Borboli & Colby 2002).

As Fuchs’ endothelial corneal dystrophy develops, the characteristic proteinaceous lesions known as guttae accumulate as excrescences on the Descemet membrane (Abbott et al. 1981). In early stages, they may produce light scatter and visual blur, when confluent and enlarged, they are not compatible with endothelial cell homeostasis and lead to cell death. This effect may be size related, with large guttae presenting an insurmountable challenge to cell monolayer formation. Gutttae typically follow a centrifugal distribution pattern with peripheral involvement in advanced disease stage (Elhalis et al. 2010). By electron microscopy, Descemet membranes in FECD show a typical additional posterior banded layer (PBL) consisting of dense fibrillary components and collagen compared with normal Descemet membranes (Xia et al. 2016). Furthermore, electron microscopy reveals a banded structure within guttae. With regard to the
ultrastructure of guttae, there are many hypotheses, but the exact mechanisms of PBL and gutta formation are unclear. One hypothesis suggests unknown processes may lead to a transformation of endothelial cells to a fibroblast-like phenotype, secreting collagen fibrils and thus forming the PBL. Focal accelerations of those transformation processes are suggested to lead to guttae (Iwamoto & DeVoe 1971; Adams et al. 1993).

State-of-the-art treatment of FECD is the endothelial keratoplasty. The predominant forms remain Descemet stripping endothelial keratoplasty (DSEK), where the diseased host Descemet membrane and endothelium are removed and replaced through donor endothelial cells and an additional stromal layer (Wacker et al. 2016), and Descemet membrane endothelial keratoplasty (DMEK; Dapena et al. 2013). First described by Melles et al. (2006), DMEK differs from DSEK by replacing the abnormal Descemet membrane and endothelium without any stromal graft leading to a significantly faster recovery of visual acuity (Price et al. 2009).

The demand for corneal grafts worldwide is continuously rising, in particular for the treatment of Fuchs' endothelial corneal dystrophy. According to recent figures, FECD is the number one indication for corneal transplantation worldwide. In 2012, 39% of all corneal grafts were used for the treatment of FECD (Gain et al. 2016). In light of anecdotal evidence suggesting some regenerative capacity of the native endothelium (Dirisamer et al. 2011), and in need of a graft-free alternative, many groups came up with a novel procedure: Descemet stripping without endothelial keratoplasty (DWEK; Moloney et al. 2015). In brief, corneal clearance is achieved following a manual descemetoablation without using any donor tissue (Shah et al. 2012; Bleyen et al. 2013). This approach is further encouraged by recent studies reporting a positive effect of rho-associated kinase (ROCK) inhibitor ripasudil following manual descemetoablation. Overall, there is strong evidence that corneal endothelial cells (CEC) have the distinct potential to regenerate or migrate after removal of the Descemet membrane in FECD.

At present, therefore, it has been demonstrated that surgical removal of guttae along with their basement membrane improves visual acuity in FECD (Shah et al. 2012; Bleyen et al. 2013; Okumura et al. 2015; Moloney et al. 2017). Once this wound is created, however, cells in the peripheral cornea must migrate across to restore a functional cell layer, with or without the encouragement of ROCK inhibition. It has also been demonstrated in vitro that this process is faster and more reliable if the underlying basement membrane (Descemet membrane) is left intact (Okumura et al. 2018; Soh & Mehta 2018). In addition, in vivo experience of DWEK surgery has demonstrated that injury to underlying stromal tissue may result in unfavourable healing response (Garcerant et al. 2019).

The surgical challenge presenting itself therefore is to determine whether it is possible to remove guttae from the posterior cornea while preserving the underlying Descemet membrane. The guttae themselves are outgrowths of protein, tightly bound to the membrane and not easily removed. An excimer laser which photoablates tissue is an ideal tool to smooth the posterior cornea profile and allow uninterrupted migration and monolayer formation of endothelial cells.

Excimer laser

All experiments were conducted with a modified ExTra ELT excimer laser (MLase AG, Germering, Germany) with a pulsed beam emitting light of 308 nm and a pulse duration of 60–120 ns. The laser was previously licensed for minimally invasive glaucoma surgery. Laser beam output at the desired destination was maintained by a fibre optical laser applicator (FIDO, MLase AG).

Corneal endothelium and guttae ablation procedure

Following epithelial abrasion, sclerocorneal rings were mounted onto a cell culture plastic with the corneal endothelium facing up. Staining of the wounded corneal endothelial area was conducted with trypan blue (VisionBlue; D.O.R.C. Dutch Ophthalmic Research Center, Zuidland, the Netherlands).
Descemet membranes were flat mounted and pinned onto cell culture plastic dishes with entomological pins (Ento Sphinx s.r.o., Pardubice, Czech Republic). To simulate physiological conditions, sodium hyaluronate (Healon® PRO; Johnson & Johnson, New Brunswick, NJ, USA) was injected between the Descemet membrane and the cell culture plastic followed by staining with trypan blue.

Excimer laser treatment included step-by-step circular guttae ablation involving the central 4 mm of the corneal endothelium and the Descemet membrane, avoiding impact on the basal membrane of the same and without direct tissue contact. In addition, in sclerocorneal rings a corridor heading to the limbus was formed to facilitate migration of corneal endothelial cells out from the peripheral endothelium. Centrally, besides a superficial ablation, a deep wound to stromal tissue was created intentionally to investigate the effect of an accidental damage to the corneal stroma. The whole procedure was conducted on three human corneas.

**Light and electron scanning microscopy**

Sclerocorneal rings and Descemet membranes were analysed and photodocumented weekly using a stereo microscope (Stemi 508; Carl Zeiss AG, Jena, Germany) following staining with trypan blue.

Before electron scanning microscopy, corneas were fixed in a cacodylate-buffered solution containing 4% paraformaldehyde (PFA) and 4% glutaraldehyde for 24 hr. Subsequent to washing with cacodylate buffer and dehydration in ascending ethanol and acetone series, the samples were critical point dried and sputter coated with gold-palladium. Images were captured with an Auriga scanning electron microscope (Carl Zeiss AG).

To assess whether recolonized corneal endothelial cells expand on wounded area, the cell area of 60 CEC of three corneas were measured in IMAGEJ (ImageJ 1.50e4; National Institute of Health, Bethesda, MD, USA). Comparison was made between regular endothelial cells in unaffected areas of the cornea and those cells colonized on the previously ablated area (for both groups: n = 30) using Student’s t-test.

**Immunohistochemistry**

For immunohistochemistry, corneas were sliced into center-involving eighth and fixed with 4% PFA for 4 hr, followed by embedding in paraffin according to standard procedures. Meridional sections of the corneas were stained with haematoxylin and eosin and analysed by light microscopy on an Axioscan microscopy (Carl Zeiss AG).

For immunostaining against the endothelial Na+/K+-ATPase, sections were hydrated and washed with 0.1 M phosphate buffer at room temperature for 10 min and blocked with 3% bovine serum albumin (BSA) and 0.1% Triton X-100 in 0.1 M phosphate buffer for 60 min followed by an incubation with anti-mouse Na+/K+-ATPase antibodies (1:50; Novus Biologicals, Wiesbaden, Germany) in 0.3% BSA and 0.01% Triton X-100 in 0.1 M phosphate buffer for 24 hr at 4°C. Following three washing cycles with 0.1 M phosphate buffer, Alexa488-coupled anti-mouse antibodies (1:100; ThermoFisher Scientific, Waltham, MA, USA) were added for 60 min. After final incubation, samples were washed three times with 0.1 M phosphate buffer 10 min each and were mounted with fluorescent mounting medium containing 1:50 4’6-di-amidino-2-phenylenedole (DAPI; Vector Laboratories Inc., Burlingame, CA, USA). Analysis was performed on an Axioscan fluorescent microscope (Carl Zeiss AG).

**Data analysis**

Quantification of endothelialized area after trypan blue staining was conducted by a semi-automated colour threshold function in IMAGEJ. Data analysis was conducted with Microsoft Excel (Microsoft Office 365, Microsoft, Redmond, WA, USA). Graphs were plotted with PRISM 8 (GraphPad Software, San Diego, CA, USA).

**Results**

**Corneal endothelial cells migrate following descemetorhexis**

To investigate migratory capacity of corneal endothelium, we conducted manual descemetorhexis only on human corneas ex vivo. Keeping them in cell culture medium supplemented with 10 µM of ripasudil for 2 days, migration of endothelial cells from the edges of the descemetorhexis into the wounded area was observed by electron microscopy (Fig. 1A,B). The corneal endothelial cells seemed to migrate across the step formed by the edge of the descemetorhexis onto corneal stroma to re-establish corneal endothelium.

**Laser ablation of guttae and Descemet membranes additional posterior banded layer**

To analyse whether the pulsed excimer laser beam can ablate guttae and the additional posterior banded layer of the Descemet membrane of patients suffering from FEDC, preparations of patients undergoing Descemet membrane endothelial keratoplasty were whole mounted on a cell culture dish and stained with trypan blue prior to ex vivo treatment. To test whether a layer-by-layer removal ablation of both diseased structures is possible, the tip of the fibre optical laser

**Fig. 1.** Electron scanning microscopy of regular human corneas following manual descemetorhexis. (A and B) Representative images of the corneal endothelium following manual descemetorhexis. The arrows point at grouped corneal endothelial cells, appearing to span migrating networks towards the stroma, the triangles show the edge of the descemetorhexis. Scale bar: 50 µm.
applicator was carefully approached until first laser effects could be observed. By using this technique, focal excrescences could be removed with a high degree of precision (Fig. 2A,B). In addition, the spongy material of the additional posterior banded layer of the Descemet membrane could be removed in a layer-by-layer manner without affecting the subjacent normal Descemet membrane (Fig. 2C,D).

**Laser ablation of corneal endothelial cells and Descemet membrane**

To further examine whether the excimer laser can ablate corneal endothelial cells without destruction of the Descemet membrane, *ex vivo* human sclerocorneal rings were stained with trypan blue before laser treatment. By carefully approaching the tip of the fibre optical laser applicator, the corneal endothelium could be cleared without removal of Descemet membrane (Fig. 3A). Only a few ruptures in the Descemet membrane were detected. In areas where a targeted ablation was wanted, the Descemet membrane was removed completely and highly organized collagen bundles of the corneal stroma were observed by scanning electron microscopy (Fig. 3B).

**Restoration of the corneal endothelium following ablation**

To test whether corneal endothelial cells can migrate onto a laser wounded area, the endothelium and in part the Descemet membrane of human *ex vivo* corneas from healthy donors was laser-ablated following trypan blue staining. To further analyse whether a corridor towards the trabecular meshwork enhances migration of endothelial cells from the periphery to the corneal centre the circumscribed wound area was extended to the periphery at the 12 o’clock position (Fig. 4A,B). Six days after treatment, several small lighter areas, which were located in a spot-like manner adjacent to the unaffected endothelium within the trypan blue stained wound, were observed indicating an initial recolonization by corneal endothelial cells under cell culture conditions (Fig. 4C). In subsequent weeks, the lighter areas extended, fused with each other and nearly completely closed after a maximum of 38 days (Figs 4D–F and 5). Intriguingly, 2 weeks after laser ablation the broadest recolonized area was observed at the 12 o’clock position suggesting that the corridor might enhance migration of corneal endothelial cells and hence recovery of the corneal endothelium (Fig. 4D).

Figure 5 indicates a re-endothelialization of the ablated area starting promptly postsurgery. Already after 6 days, corneal endothelial cells migrated towards the wounded surface. Migration continued steadily until days 13–19. Subsequently, a strong decline of the exposed ablated surface could be observed. A nearly complete closure of the wounded area was achieved after 26–38 days (Fig. 5).

As we observed a wound closure of the laser-ablated area in our *ex vivo* experiments, we investigated morphology and specificity of migrated cells by light as well as electron scanning microscopy and immunohistochemistry. On meridional sections of the...
cornea, a re-established Descemet membrane, which is lined by flat endothelial cells, was observed (Fig. 6A). No specific signal for Na\(^+\)/K\(^+\)-ATPases was detected in the corneal stroma and epithelium, whereas adjacent to the inner side of the Descemet membrane specific staining for Na\(^+\)/K\(^+\)-ATPases was observed (Fig. 6B). Further on, no obvious damage of the corneal stroma following laser treatment was detected. In line, by electron scanning microscopy a dense monolayer of flat cells with a central dome-shaped eversion was seen (Fig. 6C). Quantification of cell size demonstrates a significant 2.8-fold increase of CEC surface in the wounded area (1122.1 \(\pm\) 294.6 \(\mu\)m\(^2\)) when compared to unaffected resident cells (398.3 \(\pm\) 49.6 \(\mu\)m\(^2\); \(p < 0.001\)), suggesting that immigrated CEC expand to cover the wounded area. Overall, our data strongly suggest that the inner corneal surface is recolonized by endothelial cells.

**Discussion**

The challenge in DWEK surgery is encouraging cell migration across a bare stromal defect. ROCK inhibition has been established as a probable aid...
in this process. Both Okumura and Mehta have also suggested that an intact basement membrane favours cell migration (Okumura et al. 2018; Soh & Mehta 2018). Mehta has even proposed transfer of an acellular Descemet graft to cover the bare stromal defect created in DWEK surgery (Soh & Mehta 2018). Therefore, we chose to analyse whether it is possible to remove guttae while keeping the native basement membrane intact.

Our findings showed that in FECD Descemet membranes ex vivo, corneal endothelium and guttae could be ablated by a 308 nm excimer laser clinically used in minimally invasive glaucoma surgery. Furthermore, corneal endothelial cells were able to migrate centripetally after excimer laser ablation from the periphery to the centre of normal human corneas within less than 4 weeks ex vivo. By histology and immunostaining against Na⁺/K⁺-ATPases, we could demonstrate that corneal endothelial cells migrate on the treated area to facilitate wound closure. For the corneal endothelium, proliferation of a minor amount of CEC close to the limbal area as well as a continuous but slow centripetal migration of CECs has been proposed (He et al. 2012). In line, the significant increase of the cell area of immigrated CECs suggests cell expansion and thus the conclusion that migration seems to play a crucial role in regeneration of corneal endothelium following central ablation.

Corneal grafting and specifically DMEK remains the gold standard therapy in FECD (Dapena et al. 2009). The aim of this therapeutic approach is to remove the damaged corneal endothelium and replace it with normal donor corneal endothelial cells. Migration and regeneration of corneal endothelial cells subsequently lead to an increase of visual acuity (Dirisamer et al. 2011). Excimer laser-aided ablation of corneal endothelium in human corneas ex vivo resulted in a recolonization with corneal endothelial cells. This is in keeping with recent literature showing a similar effect of manual descemetorhexis without endothelial keratoplasty followed by regeneration of the corneal endothelium in the treatment of Fuchs’ endothelial corneal dystrophy. Still there is a disadvantage of the so-called DWEK procedure: the time until corneal clearance and thus visual improvement is significantly longer than in DMEK (up to 10 weeks postoperatively without ROCK inhibition; Huang et al. 2018). In comparison, the conducted experiments showed a corneal endothelial recolonization within 5–6 weeks in cultured human corneas. This may be due to preservation of the anterior layer of the Descemet membrane serving as a fundament for corneal endothelial cells to migrate, as can be postulated by looking at the electron microscopy. The research group around Soh et al. (2016) showed in several models in vitro that endothelial cells migrate significantly more efficient on a basement membrane than on bare corneal stroma. However, biomechanical properties of Descemet’s membrane especially in FECD are not yet completely understood (Ali et al. 2016). Furthermore, corneal endothelial cell migration started from the preformed corridors suggesting a better migration from the periphery that cannot be established by manual descemetorhexis.

Light and electron scanning microscopy suggested a viable ablation of guttae and corneal endothelium ex vivo. To our knowledge, this is a novel technique and application of excimer lasers used in ophthalmology. Currently, the removal of the Descemet membrane in posterior lamellar keratoplasty is accomplished through manual descemetorhexis (Moloney et al. 2017) including stripping off all layers of the Descemet membrane. As excimer laser ablation of the guttae-like material is done layer by layer, the anterior layer of the Descemet membrane remains and acts as a smooth scaffold for migrating endothelial cells. Furthermore, excimer lasers are already in use in ab interno trabeculotomy (Wilmsmeyer et al. 2006) and for smoothening the stromal part of donor grafts prior to posterior lamellar

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**Fig. 6.** Immunohistochemistry and electron scanning microscopy after excimer laser ablation and re-endothelialization. (A) Histological image of re-endothelialized previously ablated corneal endothelium (haematoxylin and eosin staining). Scale bar: 50 µm. (B) Immunofluorescence. Staining of endothelial Na⁺/K⁺-ATPase (green) and cell nuclei (blue). Scale bar: 50 µm. (C) Electron scanning microscopy showing dome-shaped migrated corneal endothelial cells on the wound area. Scale bar: 30 µm.
With opaque corneas. (2) Excimer laser specifications: typical excimer lasers in ablative corneal surgery emit ultraviolet light of a wavelength of 193 nm to guarantee precise cuts and a minimum of thermal distortion (Trokel et al., 1983), in comparison with the 308 nm ab interno excimer laser used in our experiments. Therefore, further investigations on the tissue response especially of the corneal guttae and Descemet membrane to different light wavelengths must be performed to optimize the ablation efficacy and minimize potential collateral damage. (3) Corneal endothelial recolonization: our results on corneal recolonization after excimer laser ablation were acquired, using healthy human corneas and under treatment with ripasudil. However, in FECD patients, the endothelial cell density is decreased and thus the recolonization potential may differ. Therefore, experiments on FECD corneas and different sizes of the ablated area are required to investigate the regenerative potential of corneal endothelium following excimer laser treatment of diseased corneas.

In summary, we could demonstrate a promising experimental approach to excise guttae and parts of the Descemet membrane by excimer laser ablation, which in turn enables corneal endothelial cells to migrate easily onto the wounded area. However, several methodological problems have to be solved in advance and in vivo studies need to be performed. Advantages of this novel technique for potential treatment of FECD might be the avoidance of a corneal graft in combination with the creation of a wound that does not present bare stroma, cannot initiate an unfavourable stromal healing response and may encourage faster cell migration over an intact basement membrane.

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