Elucidation of the migratory behaviour of the corneal endothelium

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CHAPTER 6

Improving endothelial explant tissue culture by novel thermoresponsive cell culture system

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ABSTRACT

Aim: Studying cell migration of corneal endothelial cells *in vitro* is challenging because the capacity for cell migration needs to be maintained while at the same time the tissue must remain fixed on a rigid substrate. In this study, we report a thermoresponsive culture technique designed to maintain cellular viability, and to reduce tissue handling in order to analyze *in vitro* endothelial cell migration from corneal grafts.

Materials and Methods: As a test tissue, fifteen Quarter-Descemet membrane endothelial keratoplasty (Q-DMEK) grafts were used that were embedded in a three-dimensional culture system using a temperature-reversible hydrogel and cultured over 2–3 weeks in a humidified atmosphere at 37°C and 5% CO_2.

Results: All grafts could be successfully cultured inside the thermoresponsive polymer solution for periods of up to 21 days. Using this system, cell migration could be assessed by light microscopy at fixed time intervals. At the end of the culture period, the gel could be removed from all grafts and immunohistochemistry analysis showed that endothelial cells were able to maintain confluence, viability, and junctional integrity. Some problems were encountered when using the thermoresponsive cell culture system. These were mostly structural inconsistencies during the sol-to-gel transition phase that resulted in the formation of tiny bubbles in the matrix. Additionally, areas with different viscosity resulted in optical distortions showing up as folds throughout the matrix which can persist even after several cycles of culture medium exchange. These effects had impact on the imaging quality but did not affect the viability of the explant tissue.

Conclusion: This study proves that temperature-reversible hydrogel is a very useful matrix for studying *in vitro* corneal endothelial cell migration from explant grafts and allows for subsequent biological investigation after gel removal.

Introduction

Human corneal endothelial cells form a post-mitotic layer that is not thought to proliferate *in vivo* and are known to be difficult to culture.[1–3] The cells do however retain the capacity to spread and migrate to restore a defect in the endothelium as a wound healing response. This restorative capacity is already being applied in clinical practice by new surgical techniques and the rate of cells migration is directly related to the speed of corneal clearance and postoperative recovery.[4–7]

Study endothelial cell migration *in vitro* is challenging since simply placing the explants on glass, even when coated with extracellular matrix proteins, tends to result in poor adhesion, outgrowth, and grafts lifting off the slide.[5] Corneal endothelium on Descemet membrane, once peeled off the cornea, has a well-known rolling property in fluid rendering explant culture very difficult.[8] We therefore developed a new approach by embedding the explant in a thermoresponsive gel matrix for studying endothelial cell migration from shape-adapted endothelial grafts.[9] In this paper, we describe the technical 'ins and outs' of the proposed culture system that demonstrates the ability to be remodeled by cells during migration, permeability to oxygen and nutrient growth factors, and maintaining the tissue fixed on a rigid surface.

Materials and methods

Materials

Dulbecco's phosphate-buffered saline (PBS), fetal bovine serum (FBS), L-Glutamine, ascorbic acid 2-phosphate (Asc-2P), basic fibroblast growth factor (bFGF), Dulbecco's Modified Eagle's medium (DMEM), and Calcein-AM were purchased from Sigma-Aldrich Chemistry BV (Zwijndrecht, The Netherlands). Fibronectin, collagen, and albumin (FNC) coating mix was purchased from Athena ESTM (Baltimore, MD, USA), Pen/Strep Pre-Mix from Carl Roth GmbH + Co. KG (Karlsruhe, Germany), and Mebiol® Gel (Cosmo Bio, Carlsbad, CA, USA) from Bio-Connect B.V. (Huissen, The Netherlands).

Methods

Corneas

Human postmortem corneas, ineligible for transplantation, but with an intact and viable endothelial cell layer were obtained from Amnitrans EyeBank Rotterdam. There being no objection by the donors to transplant-related research, the study adhered to the tenets of the Declaration of Helsinki and the Barcelona Principles.[10]

Tissue preparation

Fifteen Quarter-Descemet membrane endothelial keratoplasty (Quarter-DMEK) grafts were prepared as described previously.[11] Quarter-DMEK-grafts was then stored separately in organ-culture medium (CorneaMax, Eurobio) for fewer than 24 hours before chemotactic cell ability evaluation.

Preparation of Mebiol® Gel culture medium and tissue embedding process

The lyophilized thermoresponsive hydrogel is liquid at lower temperatures (4–8°C) and becomes a firmer and more gel-like matrix at the culture temperature (37°C). The gel is prepared by dissolving in 50 ml DMEM supplemented with 15% FBS, 2 mM L-Glutamine, 2 ng/ml bFGF, 0.3 mM Asc-2P, and 10,000 U-ml Pen/Strep,

refrigerated at 4°C for three hours to create a viscous solution, then carefully aliquoted to avoid air bubbles, and stored at −20°C.

For gel embedding, tissue was placed endothelial-side-up on FNC-coated glass coverslips and transferred to a 24-well plate. After embedding, Quarter-DMEK grafts were photographed daily with an AxioVert.A1 microscope with AxioCam 305 color camera (Zeiss, Oberkochen, Germany).

Immunohistochemistry

After gel removal, different structural and functional markers such as ZO-1 (tight junction protein 1 (TJP1)/ zonula occludens-1) , vimentin, and sodium–potassium pump (Na^+/K^+ –ATPase) were used to prove the feasibility of immunohistochemistry characterization after gel removal and to show the structural and functional integrity of the cells. In addition, Calcein-AM staining was performed to verify cell viability after gel removal. The staining protocols have been described previously.[5,12]

Results

Tissue embedding

For embedding the explant tissue in the gel matrix, Quarter-DMEK grafts were placed endothelial side-up on the FNC coated substrate and the thermoresponsive medium mix was added slowly, drop by drop over the center of the graft, to prevent shifting. After the graft was covered by a thin layer, it was incubated at 37°C for about 5 minutes to solidify the layer. Once firm, more medium mix was added up to a volume of approximately 700 µl. Subsequent incubation at 37°C for about 10 minutes led to a solidified gel matrix uniformly distributed over the grafts. Growth factors and nutrients were replenished by keeping the gel surface moist with 300 µl of culture medium[13] every 2–3 days.

Gel removal

All Quarter-DMEK grafts could successfully be recovered from the gel matrix by cooling the gel below the sol-gel transition temperature (<20°C). Firstly, the warm culture medium was replaced by cold fluid (PBS or DMEM) and gently aspirated 5 minutes later, removing the uppermost liquefied layer of the gel. This was performed several times until the gel was removed completely. No fixating of the graft was required before gel removal to keep it in place.

Cell migration and comparison with previous approach

Gel embedding was successful for all grafts and allowed for observation of cell migration for up to 21 days during which, cells appeared viable and continued to migrate. After 21 days, cells had not shown any change in viability and the rate outgrowth had not abated. Longer culture times may therefore be possible in contrast to our previous approach, serving as a negative control, in which the grafts were “sandwiched” between two glass slides separated by a suture wire to prevent direct compression.[5]

Immunohistochemistry

After successful gel removal, tissue could be evaluated further by immunohistochemistry and the migrated cell layer (**Figure 1A**) remained attached to the glass support in all cases. Cells showed expression of the structural

and functional markers in the confluent layer of migrating cells and on the graft itself (**Figure 1B, C, E**) after gel removal. Calcein-AM staining confirmed cell viability after gel removal (**Figure 1D**). The dashed white line outlines in (**Figure 1A**) the cell migration edge and in (**Figure 1B-E**) the Quarter-DMEK graft edge.

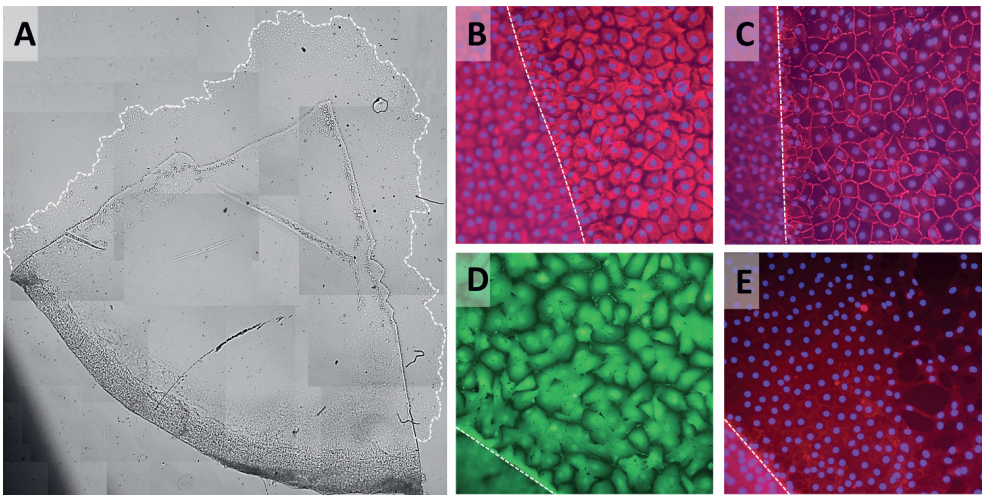

Figure 1| Example of biological research after gel removal. (A) Collage of light microscopy images (x50 magnification) to create an overview of a flattened Quarter-DMEK graft at the end of the cell migration experiment, after removing the liquefied gel at temperatures below 20°C. Expression of vimentin (B), ZO-1 (C), Calcein-AM (D), and Na^+/K^+ –ATPase (E) determined by immunohistochemistry in the confluence monolayer of cultured cells. x200 magnification.

Thermoresponsive hydrogel matrix – possible complications

Some problems were encountered when using the thermoresponsive cell culture system that can be avoided or managed. The most frequent was the formation of air bubbles inside the matrix that appeared to variable degrees for all embedded samples (**Figure 2**). Additionally, areas with different viscosity result in optical distortions showing up as folds throughout the matrix which can appear even after several cycles of culture medium exchange (**Figure 2**). These had an impact on the imaging quality but did not affect the explant tissue itself. In case of air bubble formation close to the graft, it was found that cooling the gel down to 15°C, gently aspirating the fluid and restarting the sample embedding process, could minimize the bubble. Air bubbles that formed more superficially in the gel matrix, were observed to decrease in size and shape during culture. In case of optical folds due to inhomogeneous viscosity distribution in the gel matrix, these may become more uniform in structure after few warm-RT cycles of culture medium exchange.

In 2/15 grafts, we observed graft shifting during gel removal (**Figure 2**) which resulted in a disruption of the newly formed monolayer. This tissue gliding over the solid support during gel removal can be minimized by taking care to aspirate the liquified gel as slowly as possible.

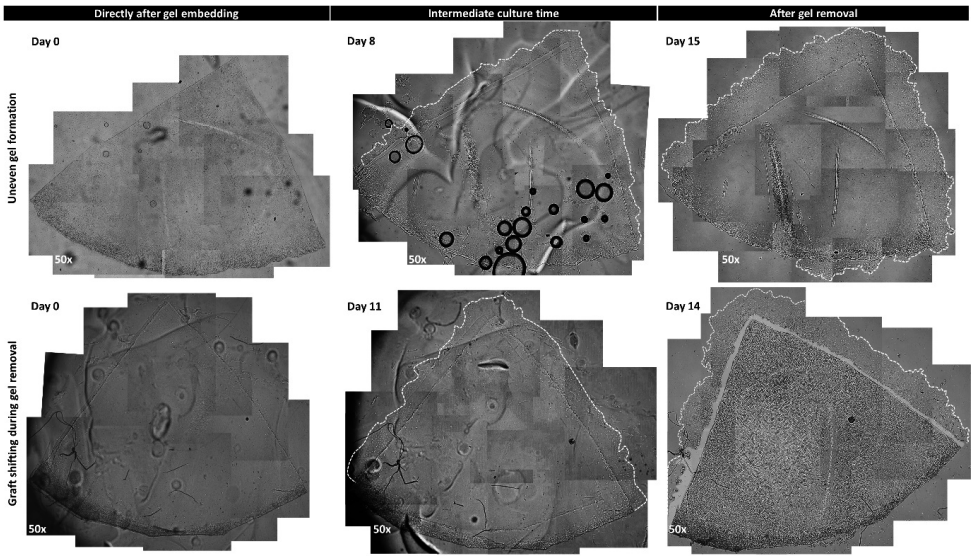

Figure 2| Complications of the thermoresponsive-hydrogel technique. The top panel displays a case of uneven gel formation: after placing the graft endothelial-side-up on FNC-coated glass coverslip (day 0, before culture) the addition of stocking gel led to small air bubble formation (day 8). In addition, minor folds throughout the matrix could be seen (day 8). Both the air bubble and folds had no impact on the graft viability during culture (day 15, after gel removal). The bottom panel shows a case of graft shifting (day 14: yellow outlined area) from its original positions (day 0) during the gel removal step. Throughout the culture time, the graft did not change its position and showed substantial endothelial cell migration (day 11) along the radial cut graft edges as outlined by the dotted white line. x50 magnification.

Discussion

With this adapted explant culture protocol based on a thermoresponsive hydrogel for *in vitro* studies on corneal grafts, we saw improved cell viability and collective cell migration which continued far longer than with the prior[5] culture system.

Grafts were kept up to 3 weeks in the hydrogel matrix and when the gel reaction was reversed, immunohistochemistry demonstrated the presence of viable cells with tightly packed morphologies. It is important to mention that on the day when grafts were recovered from the gel, cells had not shown any change in the rate of outgrowth or cell viability, therefore longer culturing times could have also been possible.

This culture system has been useful in several ways. Firstly, the consistent migration pattern observed *in vitro* helps explain the corneal clearance after corneal transplantation.[5,6,9,11]

Secondly, this method may help optimizing graft preparation to try to promote cell migration,[9] but can also be used for other purposes. This thermoresponsive cell culture system supports morphological and physiological cell changes through specific scaffold geometry and composition.[14,15] Although, other cell culture matrices provide good cell adhesion, they could in the same time make cell retrieval more difficult.[16] The temperature-reversible properties of the hydrogel presented here, on the other hand, permit cell retrieval without enzymatic treatment, which is difficult using hyaluronic acid hydrogel matrices.[17]

The question of maintaining cell polarity during cell migration in gel culture may be an issue. However, we previously demonstrated the monolayer dynamics during migration, emphasizing the difference in marker

expression between front and rear edge of the cell monolayer,[9] inferring that after migration, normal polarity can return as seen *in vivo*.

While the presented approach allows to keep the explant tissue flattened in a fixed position on the substrate, provides sufficient nutrient supply, and enables tissue retrieval at the end of the culture period, some technical challenges may occur during tissue embedding and retrieval. Occasionally, air bubbles get trapped in the matrix while adding stocking gel over the graft and inhomogeneous viscosity distribution may induce optical folds that should not be confused with folds of the tissue itself to avoid unnecessary tissue handling due to attempted repositioning.

Conclusion

In conclusion, this culturing technique based on temperature reversible hydrogel proves to be a very useful matrix for studying *in vitro* cell migration from explant tissue and allows for subsequent fundamental biological research after gel removal.

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