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Original Article

Cultivation and characterisation of the surface markers and carbohydrate profile of human corneal endothelial cells

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Abstract

Background: The study aims to characterise human corneal endothelial cell (HCEnC) cultures generated by the peel-and-digest method based on their surface protein/carbohydrate expression pattern.

Methods: Quantitative polymerase chain reaction was used to compare expression of vimentin, CD90, Cytokeratin-19, ZO-1 and Claudin 14 in cultured HCEnC and cell line B4G12 *versus* stromal cells. Fluorescence-activated cell sorting was used to assess surface protein distribution of cultured and uncultured HCEnC. Distribution of surface proteins/carbohydrates was visualised by immunofluorescent and lectin staining.

Results: Human corneal endothelial cell and B4G12 showed lower expression level for vimentin, CD90, Cytokeratin-19 compared with stromal cells; while ZO-1 was expressed in endothelial cells, Claudin 14 was detected in B4G12 only. Fluorescence-activated cell sorting analyses revealed CD166, CD47, CD44, CD54, CD73, CD90, CD105, CD106, CD112, CD146 and CD325 to be present, with CD34 to be absent from cultured HCEnC. Freshly isolated, non-cultivated HCEnCs were CD90, CD73, CD146 and CD325 positive. Carbohydrates were detected by lectins LCA, PHA E, PHA L, PSA, sWGA, Con A, RCA 120 and WGA, but cultured HCEnC showed negative for GSL I, SBA, DBA, PNA and UEA I.

Conclusion: Cultures established by the peel-and-digest method are probably not prone to stromal contamination, but the cells are likely to undergo endothelial-to mesenchymal transition as suggested by apparent morphological changes.

Key words: glycoconjugates, human corneal endothelium, immunophenotyping, lectin, surface marker.

Introduction

Establishing a method for *ex vivo* generation of viable human corneal endothelial cell (HCEnC) cultures has been a major challenge in corneal endothelial research for the last 30 years. Interdonor variability and data reproducibility issues have been the main drive to devise a standard model for isolating, cultivating and expanding HCEnCs. Commercially available SV40 immortalised cell lines such as HCEnC-B4G12 or HCEnC-H9C1 have also been used in research for their expression of functional proteins resembling primary HCEnC.[1] Although other clonal cell lines have been produced in an attempt to set up *ex vivo* models for corneal endothelial diseases,

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recent findings report a better resemblance of primary isolated cells to the *in vivo* ones compared with any cell line.[2] Corneal endothelial cells have been successfully generated from human embryonic stem cells (ESCs) as well, the latter expressing pump function-related transcripts as well as other tight junction-related proteins, such as zonula occludens-1 (ZO-1) and Na/K ATPase.[3] The ESC-derived HCEnCs could be a reproducible method for generating corneal endothelium; however, access to ESCs has been rather limited, and it has faced ethical and technical challenges as well. Currently considered a limiting factor in developed countries, HCEnCs are usually obtained from cadaveric tissue. The isolation procedure involves Descemet's membrane peeling together with the endothelium and digestion in collagenase, which yields cell sheets that are further dissociated by trypsinization.[4] The process generates a population of single cells, the morphology of which can vary from cobblestone to fibroblastoid. Presence of stromal cells cannot be excluded under such conditions. Expression of CD166/ALCAM and Peroxiredoxin-6 has been found to be an indicative marker for HCEnCs *in situ* and could be used to assess overall purity of the cell population.[5] In addition, cell surface markers such as glypican-4 and CD200 have been found to be specifically expressed on HCEnCs in comparison with corneal stroma cells (CSCs).[6] Recently, a panel of markers has been suggested for the separation of adult HCEnCs.[7] Besides proteins, each cell type has its own set of surface carbohydrates for better identification. These molecules are known to be involved in the process of cell adhesion, differentiation, development and cancer cell metastasis.[8] An impaired glycosilation profile may be associated with disease state or progression and thus be informative about the state of cells in culture.

One of the biggest concerns when expanding HCEnC is contamination by stromal cells during isolation.[5] Even so, some methods use enzymatic steps, which reportedly result in a loss of phenotype and functionality,[9] and such HCEnCs are forced to adapt to a new environment in which they are more likely to shift to a more fibroblastoid morphology, undergoing endothelial-mesenchymal transition. It is assumed that cultured HCEnCs secrete transforming growth factor 2, which induces a rearrangement of the microtubule system causing the cells to adopt a spindle-like cell morphology.[10] Because *ex vivo* cultivated HCEnCs do not need to fulfil a barrier function any longer, their first, new objective is to synthesise an extracellular matrix closely resembling 'home'. These post-mitotic cells have been shown *de facto* of being capable to divide and migrate *ex vivo*, with many groups having considered them non-functional and fibroblastic, because of their lack of expression of barrier function-related molecules in culture.[7] Different types of media and their composition have been shown to affect HCEnC morphology *ex vivo,*[11] while certain compounds including Rho kinase inhibitors have been shown to inhibit the actin cytoskeleton-related functions, hamper cell motility, proliferation, secretion and other functions, thus preserving the cobblestone morphology. There is also a growing amount of evidence suggesting that the corneal endothelium can recover from minor injuries in animals (although this is significantly different among species),[12] and progenitor-like cells from the transitional zone have been found between the trabecular meshwork and the corneal endothelium that could respond to such damage.[13]

To date, although a number of studies have presented markers for separating HCEnCs from other contaminating cells,[4] the presence of mesenchymal stem cell (MSC) markers, integrins, cell adhesion molecules combined with a surface carbohydrate/lectin profiling of cultured HCEnCs has not been performed. Our aim was to characterise primary HCEnCs from a cell surface marker (protein and carbohydrate 'fingerprint') perspective, which are isolated and cultivated *ex vivo*, using a popular method, known to yield successful cultures.[4] Such analysis can help set up a more robust marker panel for improved identification and a more standard way of expanding HCEnCs for future tissue engineering applications.

Methods

Isolation method

Collection of the tissue used in this study complied with the directive of the Helsinki Declaration and received approval from the Regional and Institutional Research Ethics Committee at the University of Debrecen, Hungary (DEOEC RKEB/IKEB 3094/2010 and 14387/2013/EKU-182/2013). All samples were collected from cadavers according to the EU Member States' Directive 2004/23/EC on tissue collection practice.[14] Cells were obtained from altogether 13 donors (62% men, 38% women, ageing 77 $\pm$ 11 years) within 24 h of death. Whole globes were sterilised using 5% povidone iodine (Egis, Hungary) and rinsed with phosphate buffered saline (PBS) for 5 min prior to dissecting the corneal button. Under a surgical microscope, the Descemet membrane and corneal endothelium were peeled off with the help of an angled crescent knife and forceps. The tissue was then digested in 1 mg/mL collagenase ($\geq$125 CDU/mg) (Sigma-Aldrich, St. Louis, MO, USA) for 2–3 h at 37°C with gentle rocking. Post-incubation, the cells were collected by centrifugation at 800 RPM for 5 min and treated with trypsin–EDTA (Sigma-Aldrich, St. Louis, MO, USA) to separate

the cells into singlets. The cells were washed and seeded on bovine fibronectin-coated (Thermo Fisher Scientific, Waltham, MA, USA) culture plates (Corning Costar, Sigma-Aldrich, St. Louis, MO, USA).

Corneal stroma cells were harvested from the remaining corneal tissue rid of the endothelium and epithelium. The central part, approximately 6–7 mm in diameter, was dissected into 5 × 5 mm^2 pieces using a surgical blade and put into 24-well culture plates (Corning Costar, Sigma-Aldrich, St. Louis, MO, USA).

Cultivation method

Commercially available corneal endothelial cell line B4G12 (Leibniz-Institut DSMZ-Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH, Braunschweig, Germany) was seeded into chondroitin-sulphate-laminin-coated 24-well culture plates (Corning Costar, Sigma-Aldrich, St. Louis, MO, USA) and fed with Human Endothelial SFM (Thermo Fisher Scientific, Waltham, MA, USA), on alternate days. Primary HCEnCs were cultured as before[15] seeded in attachment medium containing 5% foetal bovine serum (FBS) (Biosera, Kansas City, MO, USA) and 1% Antibiotic/Antimycotic solution (PAA, Pasching, Austria) in Ham's F12/M199 (1:1) for the first 48 h. Subsequently, it was changed to Maintenance medium: Ham's F12/M199 containing 5% FBS, 1% Antibiotic/Antimycotic solution, 1% insulin-transferrin-selenite (Sigma-Aldrich, St. Louis, MO, USA), 10 ng/mL basic fibroblast growth factor (Biovision, Milpitas, CA, USA) and 0.02 mg/mL ascorbic acid (Duchefa Biochemie, Haarlem, The Netherlands). Dulbecco's Modified Eagle's Medium with low concentration glucose (PAA, Pasching, Austria) was selected as culture medium for CSCs,[16,17]supplemented with 10% FBS and 1% Antibiotic/Antimycotic solution. Cells were then incubated under adhesive conditions in a humidified chamber at 37°C with 5% CO_2 tension. Cultivation media was changed on alternate days. Cultures were kept for 1–3 months. No cells used for the experiments presented herein were subcultured or passaged.

Immunofluorescent staining

Cells were seeded into 8-well chamber slides (Nunc™ Lab-Tek™ Chamber slides, Naperville, IL, USA) and grown using the cultivation method described previously. HCEnCs were fixed in 4% paraformaldehyde (Sigma Aldrich St. Louis, MO, USA) then washed with PBS and permeabilised with 0.1% Triton X-100 (Sigma Aldrich St. Louis, MO, USA) for 5 min. After another wash, non-specific binding sites were blocked with 1% bovine serum albumin (Sigma Aldrich St. Louis, MO, USA) and 3% donkey serum (Sigma Aldrich St. Louis, MO, USA) for 1 h at room temperature. Samples were incubated with the primary antibodies overnight at 4°C. Primary HCEnC cultures were stained for MSC and corneal endothelial cell markers: CD73, CD166, Collagen I and IV, Na/K ATPase, ZO-1 and proliferation marker Ki-67 (Table S2). After washing with PBS, secondary antibodies were applied and incubated for 1 h at room temperature. Nuclei were visualised by 4′,6-diamidino-2-phenylindole (Sigma). Negative controls were prepared without using the primary antibodies. Images for the cell line were captured by a BX51 Olympus microscope (Olympus, Waltham, MA, USA) (cell^F software); pictures of primary HCEnC were captured using a confocal microscope, Nikon Ti-E (Volocity imaging software, Perkin Elmer) (Nikon, Tokyo, Japan), while images were analysed by ImageJ.[18]

Reverse transcription – quantitative polymerase chain reaction analysis

An RLT buffer was applied to lyse cultured B4G12 and primary CSCs. After passing the samples through QIAshredder columns (Qiagen, Manchester, UK) to homogenise, RNeasy mini kit (Qiagen, Manchester, United Kingdom) was used to isolate total RNA. Extracted nucleic acid concentrations were determined with a NanoDrop spectrophotometer (Thermo Scientific, Wilmington, DE, USA). About 1 g of RNA was transcribed into cDNA, with Superscript III reverse transcriptase (Life Technologies, Waltham, MA, USA) using random hexamers as primers. About 1 L of cDNA was used in the Taqman assays perfomed (Applied Biosystems, Waltham, MA, USA). Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (Hs99999905_m1) was used as a housekeeping gene to determine relative expression levels of genes. CDNA was amplified on an Mx3005P polymerase chain reaction (PCR) system (Stratagene, Agilent Technologies, Santa Clara, CA, USA), and results were analysed with a real-time PCR Miner algorithm.[19]

In case of primary HCEnCs, total RNA was extracted using TRIzol Reagent (UD-GenoMed Medical Genomic Technologies Ltd., Debrecen, Hungary) according to the manufacturer's instructions, and RNA concentration and purity was measured using Nanodrop. Reverse transcription was performed using the High Capacity cDNA Archive Kit (Applied Biosystems, Abingdon, UK) with 200 ng total RNA per 20 μl RT reaction. The qRT-PCR was performed using the StepOnePlus RT-PCR system (Applied Biosystems) and Taqman Gene Expression assays following protocols from the manufacturer for genes *CD90/THY1* (Hs00174816_m1), *claudin14* (Hs 00273267_s1),

cytokeratin-19 (Hs 00761767_s1), *vimentin* (Hs 00185584_m1) and *ZO-1* (Hs 01551861_m1). Thermo cycling conditions were 95°C for 10 min followed by 40 cycles of 95°C for 15 s and 60°C for 1 min. The data were analysed by the $2^{-\Delta\Delta Ct}$ method as the fold change in gene expression relative to HCEnC, which was arbitrarily chosen as the calibrator and equals one, and GAPDH was used as housekeeping gene. All samples were run in triplicates.

GAPDH was used to determine relative gene expression levels, and then fold changes were calculated in B4G12 and primary HCEnCs *versus* primary CSCs.

Flow cytometric surface marker analysis

For immunophenotyping, primary HCEnC and CSCs were cultured in a single well of 24-well plate (1.9 cm^2) until they reached confluency (average time to confluency: 27.5 $\pm$ 3.5 days). Four donors were submitted to three-colour fluorescence-activated cell sorting (FACS) analysis. Fluorescein-isothiocyanate (FITC), phycoerithrin and allophycocyanin-conjugated primary antibodies CD146, CD47, CD112 (R&D Systems, MN, USA), CD90/Thy-1, CD34, CD54, CD73, CD105, CD106, CD44, CD325 and CD166 (Biolegend, San Diego, CA, USA) were used. Cultured cells were harvested by trypsinization and stained with the antibodies for 30 min at 4°C, then fixed in 1% paraformaldehyde. For comparison, the same procedure was used to stain freshly isolated HCEnCs at time point zero, referred to as uncultured HCEnCs, to check expression of CD73, CD90, CD44, CD146, CD166 and CD325.

Samples were analysed on a FACS Calibur cytometer (BD, Biosciences, Immunocytometry Systems), and data were analysed by Flowing Software 2.5 (Perttu Terho, Turku Centre for Biotechnology, University of Turku, Finland). More information about the antibodies used is provided in (Table S3). Hierarchical clustering was performed using the R software (version 3.3.0., R Foundation for Statistical Computing) as described previously.[17]

Surface carbohydrate staining

Human corneal endothelial cells were seeded into 8-well chamber slides and incubated with FITC-conjugated lectins (Vector Labs, Burlingame, CA, USA) in Lectin dilution buffer for 30 min at 4°C. Lectins against galactose and N-acteylgalactosamines, such as Griffonia (bandeiraea) simplicifolia lectin I (GSL I), *Dolichos biflorus* agglutinin, Peanut agglutinin, *Ricinus communis* agglutinin I (RCA 120), Soybean agglutinin (SBA), *Phaseolus vulgaris* Erythroagglutinin and *P. vulgaris* Leucoagglutinin (the latter two both against galactose) were used. Mannose and glucose were labelled by Concanavalin A (CON A), *Lens culinaris* agglutinin and *Pisum sativum* agglutinin (PSA). *Ulex europaeus* (UEA I) stained fucose and arabinose, while Wheat germ agglutinin (WGA) and its succinylated form (sWGA) binds sialic acid. Finally, Hoechst 33342 counterstaining was carried out. Surface glycoprotein distribution was visualised, and pictures were captured by an EVOS® FL microscope (Thermo Fisher Scientific, Waltham, MA, USA).

Cell viability

Cellular viability was tested by trypan blue exclusion test (Sigma-Aldrich, St. Louis, MO, USA) and Annexin-fluorescein isothiocyanate (FITC/propidium iodide) assay according to the manufacturer's protocol (MBL International, Woburn, MA, USA) and previous measurements.[20]

Statistical analysis

Mann–Whitney *U*-test was used to reveal significant differences. *P* value of less than 0.05 was considered significant.

Results

RT-qPCR analysis of B4G12 and primary HCEnC in comparison to corneal stroma cells

The relative expression of the genes previously described in the cornea was analysed by RT-qPCR in B4G12 cells and compared with that of primary CSCs (GAPDH being used as a housekeeping reference gene) (Fig. 1a). A 15-fold lower expression of *vimentin* was found in the cell line, compared with CSCs. *CD90/Thy-1* expression was expectedly more prominent in CSCs with an almost 13-fold increase in the latter, while the relative expression of *cytokeratin-19* was threefold lower in the endothelial cell line. Levels of *ZO-1* showed a 2.2-fold increased expression in the cell line, while the tight junction marker *claudin 14* was expressed 10 times more in the endothelial cells. *Claudin 10b* was not expressed on either of the cells analysed by RT-qPCR (data not shown). Having a similar expression pattern like the B4G12 cell line, the primary HCEnCs showed a 3.1-fold decrease in *vimentin* expression and a 3.2-fold decrease in *CD90/Thy-1* compared with CSCs. The expression of *cytokeratin-19* in primary HCEnCs was seven times lower than that of CSCs, while 4.25 times more *ZO-1* was expressed in HCEnCs compared to CSCs.

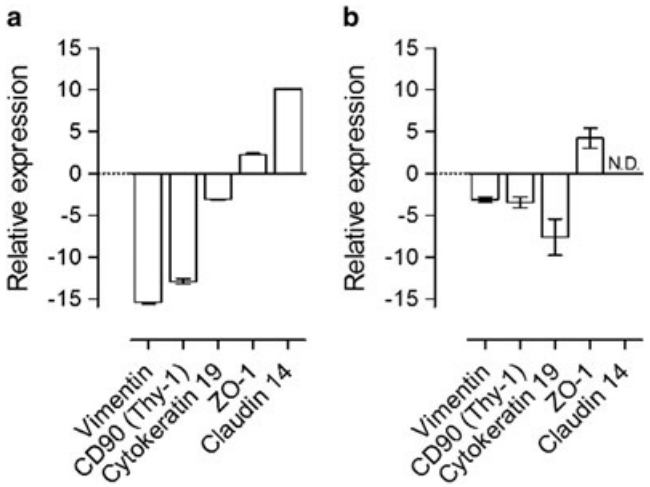

Figure 1. Reverse transcription – quantitative polymerase chain reaction analysis characterization of B4G12 (a) and primary human corneal endothelial cell (b) compared with corneal stroma cells (CSCs). Relative gene expression levels were calculated with the help of GAPDH as a reference gene, and individual gene values were normalised to that of primary CSCs. Genes with relative expression levels ±SD are shown (n = 3).

Surface protein expression pattern of primary and uncultured HCEnC

Primary HCEnC showed moderate positivity for CD73 (Fig. 2a), the signal from the stained protein being expressed diffusely within the cytoplasm. CD166 showed strong staining in the processes of the cell membranes as they assumed a stretching and forward-reaching morphology against each other (Fig. 2b). Na/K-ATPase showed positivity around the edges of HCEnCs (Fig. 2c). Staining for ZO-1 could not be detected in the intercellular space (Fig. 2d), but a dim signal was detected in the cytoplasm of the cells. Extracellular matrix components Collagen I and Collagen IV localised mainly within the cell cytoplasm (Fig. 2e,f). Sporadic expression of nuclear Ki-67 could be observed in the primary HCEnC cultures (Fig. 2g,h).

For phenotypic characterization of the cultured primary HCEnCs and uncultured HCEnCs (Table S1),

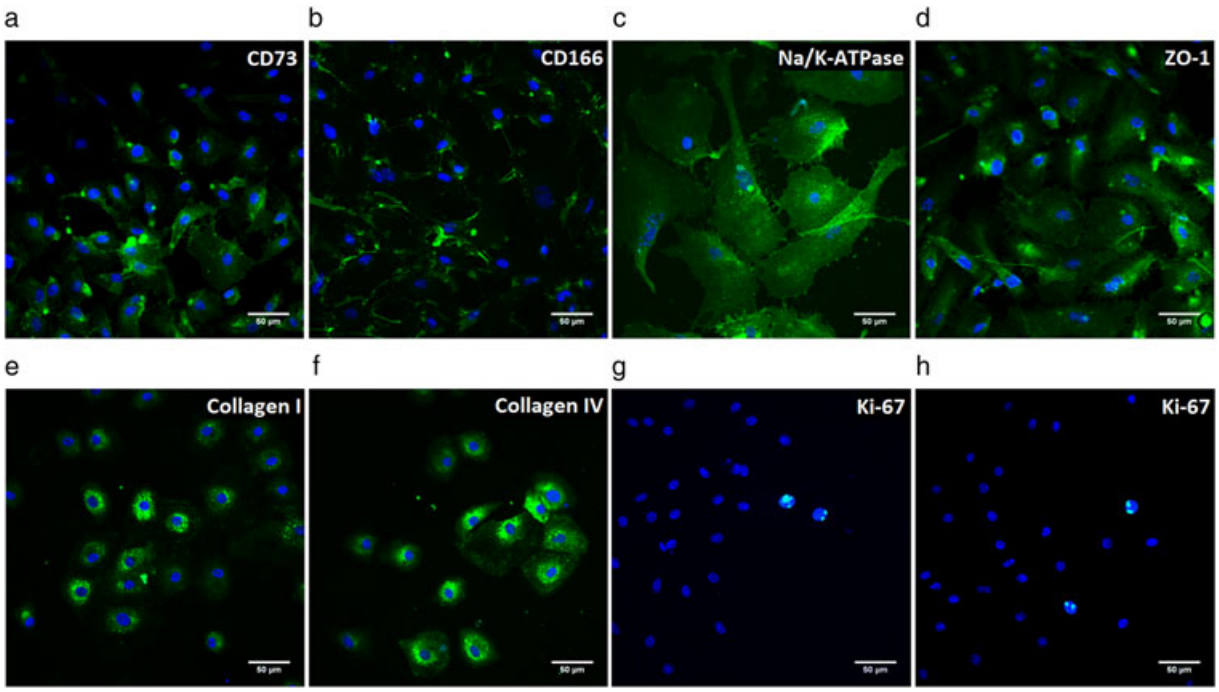

Figure 2. Immunofluorescent staining of primary human corneal endothelial cells. (a) CD73, (b) CD166, (c) Na/K ATPase, (d) ZO-1, (e) Collagen I and (f) IV and (g–h) Ki-67 staining of the cells was performed using fluorescein-isothiocyanate-conjugated antibodies. Nuclei were stained by 4′,6-diamidino-2-phenylindole (white bars represent 50 μm).

molecules representing haematopoietic and mesenchymal cell lineages, as well as integrins and proteins involved in cellular adhesion, were used. CD73 expression was found to be in 89.32 ± 1.35% of primary cultured HCEnCs compared with 16.34 ± 23.74% of the uncultured cells ($P \leq 0.001$). CD90/Thy-1 was low or absent (7.16 ± 4.46%) in cultured cells and also showed low expression in uncultured HCEnC (8.56 ± 13.26%, $P = 0.768$). Endoglin/CD105 protein was expressed by 49.52 ± 17.26% of primary HCEnCs. CD47 was found highly expressed (89.12 ± 1.92%), while CD34 – marker of haematopoietic cells – was not expressed (0.13 ± 0.13%) by the cultured HCEnCs. Cell-to-cell and cell-matrix adhesion proteins, such as CD44 (HCAM), was found on the majority of the cells with high interdonor variability (68.39 ± 13.13%) on primary HCEnCs, while a significantly lower expression, 6.68 ± 2.44% ($P = 0.041$), was observed in uncultured, freshly isolated cells. Expression of CD54 (ICAM-1) showed elevated levels (54.33 ± 8.03%); CD106 (VCAM-1) was found to be donor-dependent with high interdonor variability found on primary HCEnCs (38.08 ± 15.23%). CD146 (MCAM) expression showed similar positivity in primary HCEnC (43.02 ± 15.53%) compared with uncultured, freshly isolated cells (1.1 ± 3.15%) ($P = 0.404$). CD112 (Nectin-2) was expressed by 77.73 ± 2.17% of the cultured HCEnCs, detected as two population staining differently. CD166 (ALCAM) was present on the majority of the cells (78.93 ± 1.69%); however, significantly lower expression of the latter was observed in uncultured cells (20.20 ± 11.47%) ($P = 0.001$). N-cadherin (CD325) was found on 76.39 ± 5.68% of the primary HCEnC, showing two positive populations. It was also present on a majority (69.61 ± 7.08%) of uncultured cells ($P = 0.678$). Hierarchical clustering of the surface protein expression revealed a clear-cut difference between HCEnCs compared with CSCs. Although interdonor variability was observed in HCEnC, no relation to CSCs was found, as they formed different clusters (Fig. 3) (Table S1).

Surface lectin-carbohydrate pattern of primary HCEnC

Fluorescein-isothiocyanate-conjugated lectins were used to characterise the surface carbohydrate expression of primary endothelial cells (Fig. 4). Primary HCEnC showed positive staining for RCA lectin, recognised by terminal galactose molecules. Similarly, *P. vulgaris* leucoagglutinin was detectable, recognizing complex galactose structures. Both succinylated and non-succinylated forms of Wheat germ agglutinin (sWGA and WGA), recognizing dimer and trimer N-acetylgalactosamines, were

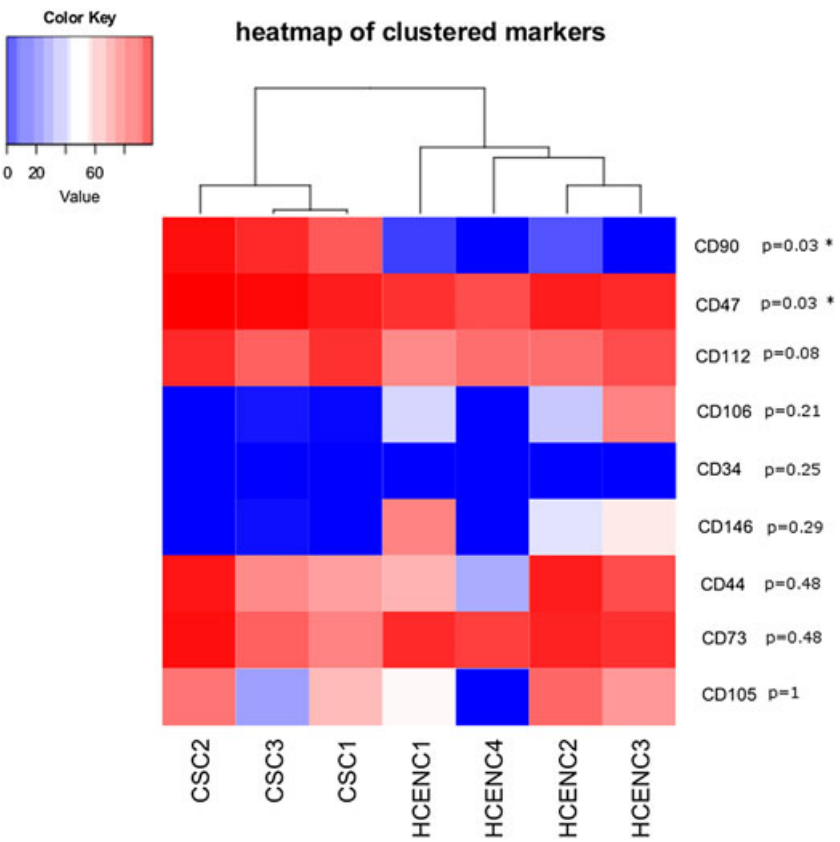

Figure 3. Heatmap showing surface proteins expressed by human corneal endothelial cell (HCEnC) compared with corneal stroma cells (CSCs). Two distinct clusters by the two cell types are seen, exhibiting the different phenotypes of the cells analysed (four donors of HCEnC and three donors of CSCs are being shown). Values 0–100 represent the percentage of positive cells for given cell surface proteins analysed by fluorescence-activated cell sorting. The *P*-values represent the statistical difference between the data obtained from HCEnCs, *versus* CSCs.

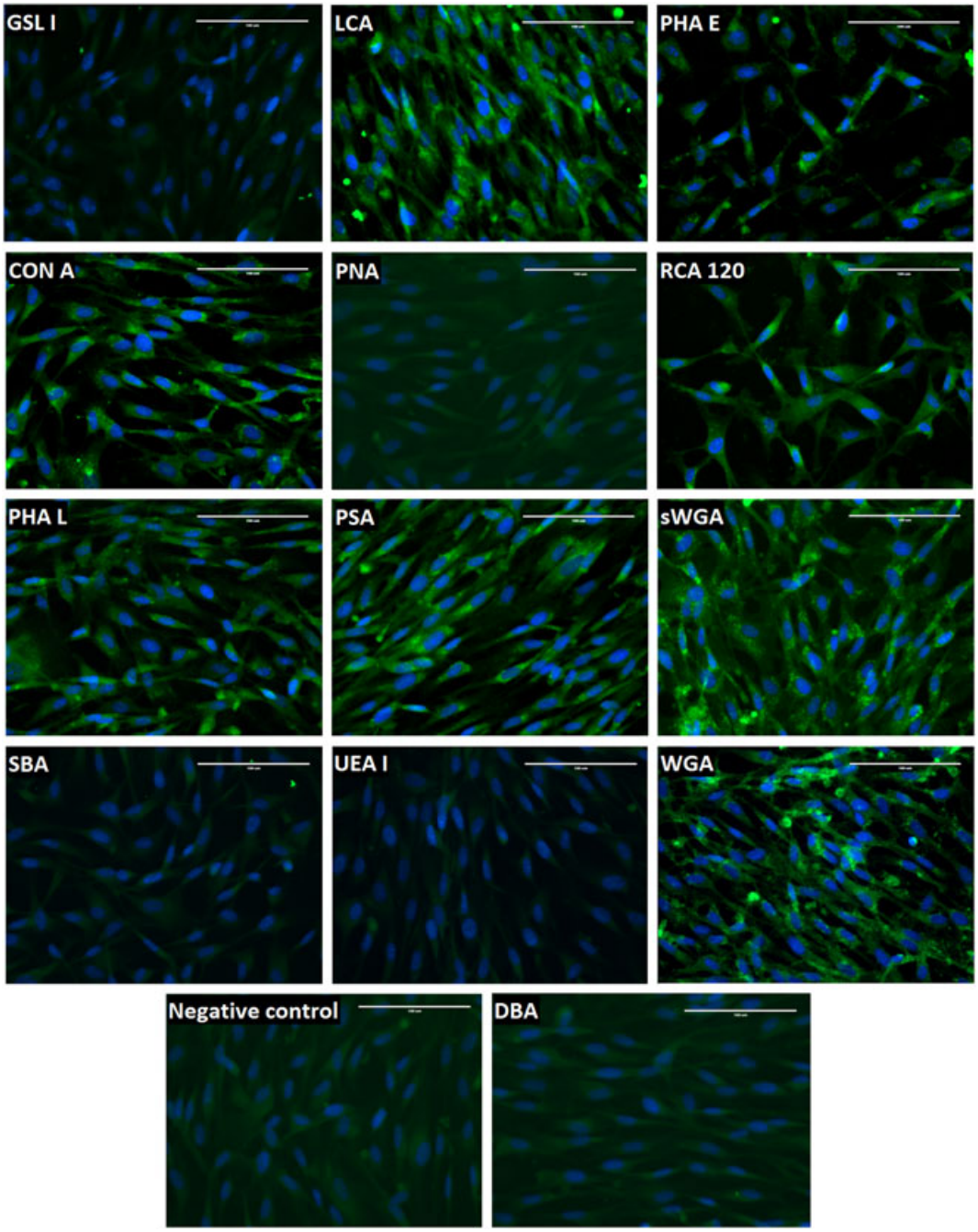

Figure 4. Surface carbohydrate staining of cultured human corneal endothelial cells (HCEnCs). Fluorescein-isothiocyanate-conjugated lectin staining was performed on primary HCEnCs. Nuclei were stained by Hoechst 33342 (white bar represents 100 μm).

detectable on the cells. Staining for *L. culinaris* agglutinin, *P. vulgaris* erythroagglutinin, PSA and CON A revealed that the cells expressed mannose and D-glucose monomers and polymers.

N-acetylgalactosamine monomers were not detectable by lectins *D. biflorus* agglutinin, SBA and Griffonia (bandeiraea) simplicifolia lectin I (GSL I). UEA I, a lectin staining L-fucose and a marker for epithelial/endothelial cells, was not detected. T-antigen-binding Peanut agglutinin was not found on primary HCEnCs.

Viability of HCEnC

The cellular viability/death assay showed that 81.84 ± 11.44% of the primary HCEnCs were viable, with presence of a moderate amount of necrotic cells (26.55 ± 15.79%) stained by propidium iodide. Apoptotic cells (4.27 ± 5.94%) stained by FITC-conjugated annexin V and double-positive/secondary necrotic or late apoptotic- (5.5 ± 4.23%) cells (Fig. 5) were detected, too. Representative sample is shown in Figure S1. Trypan blue test

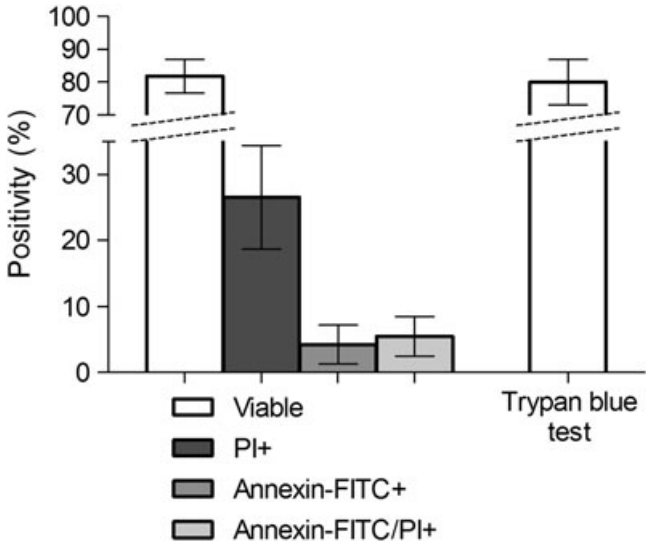

Figure 5. Cell viability in primary human corneal endothelial cells. Cell cultures were stained with propidium iodide (PI) and fluorescein-isothiocyanate (FITC)-conjugated annexin V to reveal necrotic, apoptotic or double-positive/secondary necrotic or late apoptotic cells, respectively. Viability was measured by fluorescence-activated cell sorting analysis and data represent three independent measurements on two donors. Data shown are mean ± SD of all measurements performed after 30 days of cultivation. The results of the trypan blue exclusion test are also mean ± SD (n = 4).

validated the results, showing an overall 80.01 ± 11.92% viability of the cultivated HCEnCs.

Discussion

Isolation and cultivation of primary HCEnCs has undergone many changes and improvements over the last few decades. A previously described method that is known to generate successful cultures was used here.[21] Although many studies have claimed to have found the optimal technique for isolation and cultivating HCEnCs,[4] to date, no standard method is in place for *ex vivo* cultivation of HCEnCs, with few protocols describing how to cultivate such cells over short periods of time. One study applying explanted tissue from the transitional zone between the trabecular meshwork and the corneal endothelium containing a putative stem cell population for both membranes has successfully achieved confluent cornea endothelial monolayers in a series of seven seedings over 6 months.[22] It has also been reported that media conditioned by bone marrow-derived MSCs can enhance proliferation of HCEnC and promote appearance of function-related junction.[23] A recent technique using a dual-media approach for cultivating HCEnCs reportedly produced a consistent method for expanding the cells.[15] The research, facing many challenges, ultimately aims to produce a standard method for isolation and expansion of HCEnCs for clinical application. To date, the only preclinical trial of transplanting cornea endothelial cell sheets using type I collagen and performed on monkeys proved to be unsatisfactory, and instead, single donor corneal endothelial cells have been used to treat 243 patients by a minimally invasive anterior chamber injection technique.[24] We hereby present characterization of HCEnC isolated by an enzymatic method.[4]

While the endothelial cell line showed a fast expansion rate, the isolated primary HCEnCs displayed much slower proliferation rate, which took up to 3–4 weeks of cultivation before reaching monolayer confluence. Indirectly, such a slow rate of expansion is reportive of the purity of the cultures, because fibroblasts are known to quickly overtake or outgrow other cell types *in vitro*.[25]

We have previously shown a clearly differential gene expression of limbal epithelial stem cells compared with corneal stroma-derived mesenchymal-like stem cells.[17] In the present study, the gene expression analysis by RT-qPCR revealed that the cell line and the primary HCEnCs had similar expression patterns, which clearly differed from that of CSCs. Because contamination of HCEnCs by CSCs is a main concern when isolating HCEnCs, the higher expression of cell-to-cell adhesion and cell junction genes in HCEnCs compared with CSCs further supports the fact that our isolated HCEnCs are pure or free from stromal cells. The intermediate filament *vimentin* was expressed much higher in the CSCs as opposed to the cell line, possibly demonstrating the absence of endothelial-to-mesenchymal transition in the B4G12 cells. However, presence of this protein has been demonstrated in histological sections of corneal endothelium *in situ* and in cultured HCEnCs as well.[26,27]

The expression levels of tight junction genes *ZO-1* and *claudin 14* were also confirmed in the cell line and the primary HCEnCs; interestingly, *claudin 10* was not detectable by qPCR in the latter cells, which differed from the data obtained by immunofluorescent staining of the protein, and also, not in line with the findings of others.[28] Other groups have reported detecting *claudin* expression in B4G12 cells, which differed from that of primary human endothelium. They also found interindividual variations.[28] The immortalised, transformed nature of the cell line as well as the *ex vivo* cultivation of HCEnCs may have contributed to such differences. According to another transcriptome analysis of the cell line *versus* primary and *ex vivo* cultured corneal endothelial cells, B4G12 represents a distinct type of cell, thus not being an optimal model for corneal endothelial studies.[2] Future clinically oriented experiments should therefore focus on using primary HCEnCs, possibly obtained from cadavers or other sources such as embryonic or induced pluripotent stem cells.

Characterization of the surface marker profile of corneal endothelial cells is important to determine the purity as well as the source of these cells. It has been claimed that high expression levels of the markers CD98, CD166 and CD340 are characteristic of a functional corneal endothelial phenotype, while cells bearing CD9, CD49e, CD44 and CD73 are fibroblastic, non-functional endothelial cells, when assayed by FACS.[7] Our FACS analysis revealed that majority of the cultured cells express CD73 and CD44, while CD90 was absent or present in low levels (<10%). Interestingly, the freshly isolated, non-cultivated HCEnCs also expressed CD73 and similarly low CD90 expression compared with the cultured HCEnCs.

Up-regulation of the hyaluronate receptor CD44 has been described following epithelial injury in mice[29] and has been detected in normal human colorectal cells in the proliferative regions or crypts.[30,31] CD44 has also been detected at the basal layers of corneal epithelium, in corneal keratocytes and in cornea stromal cells,[17] and absent in the endothelium of normal human corneas. Nonetheless, CD44 has also been detected in endothelial cells from the remaining parts of Fuchs dystrophy, bullous keratopathy and late stages of graft rejection, indicating an association with loss of integrity of the endothelial layer, while it has not been detected in conditions such as keratoconus, Meesmann and lattice dystrophy, where the endothelium is usually unaffected. In the former conditions and in culture, the cells need to expand and/or migrate in order to cover the surface and to maintain a uniform endothelial barrier, thus losing their original functions while adapting to the new circumstances. An association between CD44, injury and compensatory response has been previously described by others.[32] More than half of our cultured primary HCEnCs expressed CD44, possibly representing a population of cells possessing a migratory, proliferative and regenerative capacity. In comparison with freshly isolated, non-cultivated HCEnCs, the expression of CD44 appears to be higher in the *ex vivo* expanded cells. CSCs, on the other hand, are known to express CD73, CD90 and CD105.[17] Low expression of CD90 in our cultures demonstrates a cell population free or very low on CD90 expressing cells – possibly CSCs. CD90 expression of HCEnC appeared not to be elevated or changed significantly after isolation as well.

Because there is no official consensus on a definitive panel of surface molecules to identify HCEnCs, we propose supplementing the presently known fingerprint of surface marker proteins on HCEnCs, with a panel of positive surface carbohydrates for better identification and characterization of these cells.

Studies with corneal epithelial cells have reported that the expression levels and distribution of glycoproteins on the cell surface play a crucial role in the regeneration the layer,[33,34] and a fingerprint of carbohydrate molecules present on the cornea limbal epithelial stem cells has also been described.[20] Interestingly, the lectin staining of surface carbohydrate molecules on HCEnC showed similarities with CSCs,[17] probably because of their proximity or sharing of the location in the cornea *in vivo*.

The levels of glycoconjugates can be altered in different diseases affecting the cornea.[35–37] Pax-6 mutant mice demonstrated a disturbed glycoconjugate function. The authors speculated that there is either an adhesion molecule or growth factor receptor conjugated to -N-acetylglucosamine[1–4] glucose and D-mannose and/or -D-glucose, which blocked by WGA, and Con A leads to an elongated, insufficient response to injury in epithelial cells, *in vitro*.[34] Several studies have described the lectin binding pattern of animal- and human-cornea-derived *in situ* endothelial cells specimen; in the uninjured rat cornea, cells could bind to lectin WGA, Con A, RCA, while not to GSL I, SBA and UEA I[36,38]; GSL I B4 was found to be a specific marker of bovine corneal endothelium, while its expression in humans was restricted to individuals with B blood type[35,39]; Con A, RCA-120, GSL I, WGA and PHA were found to be present in the basal corneal epithelial cell layers, while the Descemet's membrane stained for Con A, PHA and PSA in frozen corneal sections.[40] It is known that during wound repair, corneal endothelial cells respond to injury by exiting from the G_0 phase and up-regulation of DNA synthesis around affected regions 24 to 66 h following injury, when mitosis and migration happen simultaneously.[41–43] More recent reports have described a G_1 arrest of the cornea endothelial cells *in vivo*, while maintaining proliferative capacity.[44] In intact corneal endothelium, SBA was found to be negative, but reportedly, injured endothelium showed SBA binding adjacent to wounded areas after 24 h. Expression of the SBA-bound glycoconjugate was completely abolished after 72 h.[36] A role of SBA in the re-organization of actin skeleton in wound-repair was also suggested.[45] The same group hypothesised that appearance of SBA-bound protein might act to re-establish cell-to-cell interaction, as it was expressed in cells under stress, such as explantation (organ culture) or disease, in order to conserve the integrity of the endothelial cell layer.[46] SBA was also found up-regulated in endothelial cells of keratoconic corneas. This suggests a relationship of surface carbohydrates in the structural integrity of the cornea.[47] In our study, primary HCEnCs did not exhibit any SBA binding, which might be explained by a restored integrity of the endothelial monolayer, even though the cells retained a fibroblastoid morphology.

In conclusion, our data show that a popular method widely used for the expansion of HCEnC produces cells that express markers associated to the corneal endothelial phenotype. Distribution of the proteins and carbohydrates shows a population of cells adapting to a new environment, while attempting to restore integrity of the original tissue. Even so, these slowly proliferating cells slowly undergo changes that will compromise their original functions. Perhaps, reverting the cells back without the use of cell-altering substances, or isolating them in a manner so that they do not change drastically, will enable future basic and applied research by isolating and propagating the HCEnC for clinical purposes. Clinical trials with HCEnCs are much needed to reach safety and efficacy of the cell therapy awaited in the near future.

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Supporting information

Additional Supporting Information may be found in the online version of this article at the publisher's web-site:

Figure S1 Representative results from the cell viability/death analysis by FACS.
Figure S2 Histograms showing surface marker analysis of cultured (A) and uncultured (B) HCEnCs.
Table S1 Surface marker profiling of primary HCEnCs, CSCs and uncultured HCEnCs (n = 4, n = 3, n = 3, respectively) by FACS analyses.
Table S2 Details of the antibodies used for immunofluorescent staining.
Table S3 List of antibodies used for the FACS analysis.