Two Clonal Cell Lines of Immortalized Human Corneal Endothelial Cells Show either Differentiated or Precursor Cell Characteristics

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Human corneal endothelium • Cell line • Serum-free cultivation • Clonal growth • Differentiation

Abstract
Access to primary human corneal endothelial cells (HCEC) is limited and donor-derived differences between cultures exacerbate the issue of data reproducibility, whereas cell lines can provide sufficient numbers of homogenous cells for multiple experiments. An immortalized HCEC population was adapted to serum-free culture medium and repeated cloning was performed. Clonally grown cells were propagated under serum-free culture conditions and growth curves were recorded. Cells were characterized immunocytochemically for junctional proteins, collagens, Na,K-ATPase and HCEC-specific 9.3.E-antigen. Ultrastructure was monitored by scanning and transmission electron microscopy. Two clonal cell lines, HCEC-B4G12 and HCEC-H9C1, could be isolated and expanded, which differed morphologically: B4G12 cells were polygonal, strongly adherent and formed a strict monolayer, H9C1 cells were less adherent and formed floating spheres. The generation time of B4G12 cells was 62.26 ± 14.5 h and that of H9C1 cells 44.05 ± 5.05 h. Scanning electron microscopy revealed that B4G12 cells had a smooth cell surface, while H9C1 cells had numerous thin filopodia. Both cell lines expressed ZO-1 and occludin adequately, and little but well detectable amounts of connexin-43. Expression of HCEC-specific 9.3.E-antigen was found commensurately in both cell lines, while expression of Na,K-ATPase α1 was higher in H9C1 cells than in B4G12 cells. B4G12 cells expressed collagen IV abundantly and almost no collagen III, while H9C1 cells expressed both collagens at reasonable amounts. It is concluded that the clonal cell line B4G12 represents an ideal model of differentiated HCEC, while H9C1 may reflect features of developing or transitional HCEC.
Introduction

The development of methods for cellular applications in regenerative therapies requires, in most cases, to work in a model system such as cell lines in order to understand the underlying cellular mechanisms, but also to define necessary technical conditions. Broad variations within an experimental setup due to the influence of donor-derived differences between primary cell cultures may aggravate obtaining reliable data within a reasonable time. For this reason, many researchers prefer cell lines as models to study typical features of specific cell types as well as their biological functions. Access to resources for normal human tissue is restricted depending on applicable legislation and availability. Therefore, cell lines that show a substantial similarity with the primary cell counterparts regarding cell type-specific characteristics can be used as an alternative, for example, in order to establish protocols for cellular therapies. For that reason, our group established an immortalized cell population of human corneal endothelial cells (HCEC) [Bednarz et al., 2000]. These cells form a functional monolayer when transplanted onto denuded donor corneas [Aboalchamat et al., 1999], have recently been characterized regarding electrophysiological properties [Mergler et al., 2003] and apoptotic mechanism [Thuret et al., 2003], and were successfully used to construct human corneal organotypic equivalents [Reichl et al., 2004; Zorn-Kruppa et al., 2005] or transferable cell sheets [Nitschke et al., 2007].

Corneal endothelial cells (CEC) show different characteristics depending on their localization within the cornea (that is, peripheral or central) and can be divided into subpopulations [Bednarz et al., 1998]. The immortalized HCEC population that was previously established by our group is derived from whole corneal endothelium, and as such represents a variety of CEC subtypes. Due to this, the cell population displays a heterogeneous morphology, comprised of small polygonal and strongly adherent cells to larger and slightly elongated cells that are less adherent. However, to obtain reliable and reproducible data with a minimum of experimental effort, it is advantageous to work with a rather homogenous cell population to exclude broad variations as a result of different influential factors derived from subpopulations or interindividual discrepancies derived from different donors. Such homogenous populations are at best clonally grown, therefore, we sought to establish clonal cell lines of HCEC. In this study, we subjected the immortalized HCEC population to a 2-step subcloning procedure after adapting the cells to serum-free growth conditions, and characterized the obtained clonal cell lines morphologically. We herein present 2 clonally grown cell lines of adult HCEC with different properties.

Materials and Methods

Cell Cultivation and Cloning of Cell Lines

The immortalized adult HCEC population previously established by SV40 transfection [Bednarz et al., 2000] was used. Cells were grown on T25 culture dishes coated with 1 mg chondroitin-6-sulphate (Sigma) and 10 µg/ml laminin ( Sigma). The cells, which were originally grown in medium F99 (Ham’s F12 Nutrient Mixture/Medium 199) supplemented with 5% FCS, 10 ng/ml bFGF, 20 µg/ml ascorbic acid and 20 µg/ml insulin, were adapted to serum-free conditions using Human Endothelial-SFM (Gibco Invitrogen) supplemented with 10 ng/ml human recombinant bFGF (Gibco Invitrogen) over 3 passages before cloning. Cells were passaged using trypsin/EDTA (0.05/0.02%; Gibco Invitrogen) after 2 min preincubation with enzyme-free cell dissociation buffer (Gibco Invitrogen) at 37°C. Enzyme activity was quenched by protease inhibitor cocktail (Complete™ Protease Inhibitor Cocktail; Roche Diagnostics; 1 tablet/50 ml medium).

For cloning, cells were seeded at a density of 0.3 cells/well on two 96-well plates. Cells were cultured with serum-free medium as described above and the plates were viewed by phase contrast microscopy every other day for over 6 weeks. In 10 wells, clonally growing cells were observed. These were separately trypsinized, replated at a density of 0.3 cells/well on 96-well plates and cultured as described above. After another 15 weeks, clonally grown cells were observed in 2 wells. These were separately trypsinized and further cultivated for cell propagation.

For recording growth curves, cells were seeded at 1,000 cells/cm² on 12-well dishes and cultured under serum-free conditions. Every 1–2 days, cells from 1 well were trypsinized and counted, and population doublings and generation time were calculated. The resulting growth curves were calculated as the means ± SD according to the Gaussian distribution from 3 repetitive experiments.

Immunocytochemistry

For immunocytochemistry, cells were cultured on chamber slides (glass bottom slides; BD Falcon) as described above. Subconfluent to confluent cultures on chamber slides were washed once with PBS with Ca²⁺ and Mg²⁺, fixed with cold methanol for 20 min at 4°C, air dried and stored at ~20°C until use. Before staining, the slides were permeabilized with PBS containing 0.2% Triton X-100 and washed 3 times with PBS plus 0.5% BSA. Cells were incubated with the primary antibodies (table 1) diluted in PBS plus 0.5% BSA at room temperature for 60 min. After washing, the bound primary antibody was detected using FITC-conjugated secondary antibody (1:500; DakoCytomation) for 30 min at room temperature in the dark. Slides were then washed in PBS, counterstained with DAPI (1:5,000; Sigma) for 3 min, rinsed with distilled water, and mounted in Pro Long Gold antifade reagent (Molecular Probes Invitrogen Detection Technologies). Slides were examined under a Zeiss LSM 510 META laser scanning confocal microscope equipped with a Zeiss filter set 9 for detection of FITC.
**Electron Microscopy**

For transmission (TEM) and scanning (SEM) electron microscopy, cells were grown on thermanox cover slips to confluence. Cells were then fixed in 2.5% glutaraldehyde and washed in 0.1 M cacodylate buffer for about 30 min. For TEM, the fixed samples were osmicated with 1% osmium tetroxide, dehydrated in ascending ethanol concentrations (30–100% in 6 steps), and embedded in a 1/1 mixture of epoxy resin and ethanol for 2 h. After 2 h, fresh epoxy resin was added to the samples and they were left overnight. Then, the resin was polymerized for 2 days at 60 °C. Ultrathin sections were cut and contrasted with 3% uranyl acetate and lead citrate. Samples were visualized in a Zeiss EM906. For SEM, the fixed samples were washed in 0.2 M cacodylate buffer, dehydrated in ascending ethanol concentrations (30–100% in 6 steps), critically point dried and sputtered with gold particles. Samples were visualized in a Leo 430 scanning electron microscope.

**Results**

**Morphology and Growth Behavior**

The subcloning procedure yielded 2 clonal cell lines of immortalized HCEC, designated HCEC-B4G12 and HCEC-H9C1. Both could successfully be propagated under serum-free conditions. The clonal cell lines differed markedly in their morphology when grown on chondroitin sulphate/laminin-coated culture dishes. B4G12 comprised small polygonal cells that grew in a strict monolayer formation. The cells were strongly adherent to their substratum and had distinct cell borders (fig. 1a and b). In contrast to the mother cell line, these cells did not produce floating daughter cells when they became confluent, but reduced their size to maintain a monolayer (fig. 1a and b). Proliferation slowed down as they became confluent, but the cells did not show strict contact inhibition.

In contrast, cells of the clonal cell line H9C1 grew as single round to elongated cells in a network-like formation or aggregated in sphere-like formations, and showed only weak adherence to their substratum (fig. 1d and e). During the cultivation phase, cells did not become confluent, but spheres increased in size. These spheres as well as single daughter cells lifted up and floated in the medium. When individualized and replated, cells from spheres readily attached and continued to proliferate, and eventually formed new spheres. Sphere formation was only observed when cells were grown on chondroitin sulphate/laminin-coated culture dishes under serum-free conditions, but was not observed when cells were grown in the original, serum-supplemented HCEC growth medium based on F99.

The mean generation time of the cells was calculated as 62.26 ± 14.5 h for B4G12 cells (fig. 1c) and 44.05 ± 5.05 h for H9C1 cells (fig. 1f), when seeded at 1,000 cells/cm². Cells of the clonal line B4G12 had a markedly longer lag phase (approximately 6 days) than cells of the clonal line H9C1 (approximately 3 days) when seeded at 1,000 cells/cm².

**Immunostaining**

Both clonal cell lines produced collagens and deposited them in their extracellular matrix (ECM). Of the collagens tested, the major difference observed was that B4G12 cells produced mainly collagen IV and almost no collagen III (fig. 2b and c), while H9C1 cells seemed to produce collagen III in abundance and less but still reasonable amounts of collagen IV (fig. 2e and f). It is worth mentioning that, although both cell lines deposited only little amounts if any of collagen I in their ECM (fig. 2a and d), the collagen pattern produced by B4G12 cells corresponds to that of primary HCEC or healthy HCEC in situ more closely than that of H9C1 cells, which resembles that of transitional HCEC found in retrocorneal membranes.

In addition, both cell clones expressed the junctional proteins ZO-1 (fig. 3a and f), occludin (fig. 3b and g) and connexin-43 (fig. 3c and h) appropriately at their lateral cell borders, giving rise to the assumption that they can exhibit a proper pump function according to their in vivo

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**Table 1. Primary antibodies used for immunocytochemistry**

<table>
<thead>
<tr>
<th>Antigen</th>
<th>Clone</th>
<th>Supplier</th>
<th>Dilution</th>
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<tr>
<td>Connexin-43</td>
<td>RN26</td>
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<td>Occludin</td>
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<td>Transduction Laboratories</td>
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<td>Transduction Laboratories</td>
<td>1:150</td>
</tr>
<tr>
<td>Na,K-ATPase α1</td>
<td>9-5A</td>
<td>Abcam</td>
<td>1:200</td>
</tr>
<tr>
<td>Collagen I</td>
<td>1-8H5</td>
<td>Acris Antibodies</td>
<td>1:100</td>
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<td>Collagen III</td>
<td>III-53</td>
<td>Acris Antibodies</td>
<td>1:100</td>
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<tr>
<td>Collagen IV</td>
<td>NLI/53</td>
<td>Acris Antibodies</td>
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counterparts. Both clonal lines showed a homogenous expression pattern of Na,K-ATPase (fig. 3d and i) with the signal intensity being higher in H9C1 cells. The localization of Na,K-ATPase was found to be predominantly in the cytoplasm, but could also be seen accumulating at the lateral cell borders. The corneal endothelium-specific 9.3.E-antigen (fig. 3e and j) was similarly expressed in both clonal cell lines.

**Electron Microscopy**

Using electron microscopy, differences in the ultrastructure of the 2 clonal cell lines became visible (fig. 4). TEM revealed that cells of the clonal line B4G12 grew in close contact to each other and showed only few membrane protrusions. Nuclei and mitochondria appeared normal and the rough endoplasmic reticulum was prominent (fig. 4a–c). In contrast, cells of the clonal cell line H9C1 did not grow that close to each other and possessed numerous filopodia. The cells also had a noticeable number of vesicles. Mitochondria and rough endoplasmic reticulum appeared normal (fig. 4d–f). Using SEM, cells of the B4G12 line appeared rather flat and tightly adhered to their substratum, and had a rather smooth cell membrane with only few protrusions (fig. 4g–i), while cells of the H9C1 cell line showed numerous filopodia and microvilli (fig. 4j–l). H9C1 cells were rounded up, often aggregated in small groups, and appeared less adherent to their substratum than B4G12 cells. Overall, H9C1 cells appeared more proliferative and less differentiated than B4G12 cells.

**Fig. 1.** Growth curves and cell morphology of the 2 clonal HCEC lines in serum-free culture medium on chondroitin sulphate/laminin-coated culture plates. a, b Clone B4G12. The cells were small, polygonal, and grew in a strict monolayer formation. When reaching confluence, the cells became markedly smaller in size. Phase contrast; scale bars = 160 μm. c Growth curve. Cells were seeded at a density of 1,000 cells/cm², mean generation time was 62.26 ± 14.5 h. d, e Clone H9C1. The cells grew as single round or elongated cells or aggregates in sphere-like formations. Phase contrast; scale bar = 320 μm (d), scale bar = 160 μm (e). f Growth curve. Cells were seeded at a density of 1,000 cells/cm², mean generation time was 44.05 ± 5.05 h.
Discussion

Cell lines are suitable model systems for basic research in cell biology or in developing methods for cellular applications in regenerative therapies. The HCEC cell population established by Bednarz et al. [2000] is a great advantage in the field of HCEC research, but it is a heterogeneous cell population derived from whole corneal endothelium, with its heterogeneity representing the entirety of HCEC populations, including peripheral and

Fig. 2. Immunostaining for collagens: B4G12 cells (a–c), H9C1 cells (d–f); collagen I (a, d); collagen III (b, e); collagen IV (c, f). B4G12 cells produce collagen IV as their main collagen and no detectable collagen III, while H9C1 cells produce both collagens. Collagen I was barely detectable in both cell lines. Scale bars = 20 μm.

Fig. 3. Immunostaining for HCEC-typical proteins: B4G12 cells (a–e); H9C1 cells (f–j). Staining for the junctional proteins ZO-1 (a, f), occludin (b, g) and connexin-43 (c, h) displays a regular distribution at the intercellular contacts in both clonal cell lines, with connexin-43 also being observable in the cytoplasm. Na,K-ATPase (d, i) is located predominantly at the cytoplasm and accumulates at the lateral cell borders at intercellular contacts in both cell lines, with staining intensity being higher in H9C1 cells than in B4G12 cells. The HCEC marker 9.3.E-antigen (e, j) is distributed regularly at the lateral cell borders as described in the literature. Scale bars = 20 μm.
Central cells. In order to precisely characterize cellular features and functions, and with respect to the known differences between central and peripheral CEC, homogeneity of such a cell line seems advantageous. It is known from studies with primary HCEC that centrally derived cells lack a proliferative capacity, while cells from the corneal periphery show some proliferative capacity [Bednarz et al., 1996, 1998; Whikehart et al., 2005]. It was concluded from these results that central HCEC are terminally differentiated, postmitotic cells, while the periphery may

Fig. 4. Transmission electron micrographs revealed that cells of both clonal lines had normal cell organelles. B4G12 cells (a–c) seemed to possess more prominent endoplasmic reticulum and mitochondria than H9C1 cells (d–f) and had a smooth cell surface, while H9C1 cells had numerous microvilli and filopodia. Scale bars = 10 μm (a, d), scale bars = 2 μm (b, c, e, f). Scanning electron micrographs confirmed that B4G12 cells (g–i) were small with a rather smooth cell surface, were very adherent to their substratum and had close intercellular contacts. In contrast, H9C1 cells (j–l) had numerous microvilli and filopodia, and were only loosely attached to their substratum and to each other, as could be recognized by their roundup shape. Scale bars = 10 μm.
habor progenitor or transient amplifying cells of the corneal endothelium. Recent studies report on sphere formation of HCEC in vitro and that these spheres contain progenitor cells of HCEC [Yokoo et al., 2005], with the majority of HCEC progenitors residing in the corneal periphery [Yamagami et al., 2007]. The morphological differences between the 2 clonal cell lines described in this work allow the conclusion that they may reflect model lines for central and peripheral HCEC. It seems likely that B4G12 cells are centrally derived, since they grow very adherent and in an almost strict monolayer formation, similar to well-differentiated HCEC in situ. H9C1 cells may be derived from the periphery, because they have a higher proliferative capacity, show only limited adhesion to their substratum and tend to grow in a sphere formation similar to that described by others [Yokoo et al., 2005; Yamagami et al., 2007]. This conclusion is further supported by ultrastructural findings obtained by electron microscopy. As demonstrated, H9C1 cells form microvilli and filopodia, and grow in weakly adherent cell aggregates. It is known from other cell types, for example urothelial cells [Erman et al., 2006], that they possess microvilli only during certain developmental stages, but not when they have achieved a fully differentiated state. In contrast to H9C1 cells, cells of the B4G12 line have a smooth cell surface without protrusions and develop close intercellular contacts. However, it has never been investigated in HCEC, whether less differentiated or transdifferentiated cells differ from fully differentiated cells on an ultrastructural level.

An important feature of CEC is their ability to synthesize and deposit collagens into their ECM. Collagen synthesis was investigated previously in corneal endothelium in vivo and in vitro in several species at different developmental stages [Ben-Zvi et al., 1986; Kay et al., 1993; Levy et al., 1995; Funderburgh et al., 2003; Esquenazi et al., 2006]. It could be shown that collagen IV is the main collagen produced by normal CEC and is also the main component of Descemet's membrane, while collagen III can be detected in the ECM of normal bovine CEC, but is barely detectable in the ECM of normal rabbit CEC [Kay et al., 1993]. Only few groups reported on collagen III in the human corneal endothelium; for example, Ben-Zvi et al. [1986] state that collagen III was detected in the human cornea during embryonic development in Descemet's and Bowman's membrane, but could not be detected in the central cornea anymore after the 27th week of gestation. On the other hand, other groups reported the finding of collagen III during corneal wound healing [Funderburgh et al., 2003; Esquenazi et al., 2006] or in pathologic matrix deposits in the diseased corneal endothelium [Levy et al., 1995]. It is known from histological examinations of retrocorneal fibrous membranes that HCEC can transdifferentiate towards a fibroblastoid phenotype. It could be shown in a rabbit model that such transdifferentiated HCEC are characterized by an induction of collagen I synthesis and upregulation of collagen III synthesis in contrast to normal CEC [Kay et al., 1993]. But besides sites of tissue remodelling, collagen III can also be found in the corneal limbus, a site for progenitor cells of the corneal epithelium, in association with blood vessels [Ben-Zvi et al., 1986]. Since the 2 cell lines described here do not synthesize collagen I but deposit collagen IV in their ECM, their phenotype can be apprehended as normal, even though cells of the H9C1 cell line do not grow in a typically polygonal monolayer formation. With regard to the aforementioned findings by other groups, the synthesis of collagen III by H9C1 cells may be an indicator of their possibly transitional or less differentiated phenotype. The detection of collagen III in retrocorneal membranes as described by Levy et al. [1995] and Kay et al. [1993] indicates a transition of CEC from a neuroectodermal to a mesenchymal phenotype under pathologic conditions. However, with respect to findings by Yokoo et al. [2005], who reported the expression of mesenchymal markers in HCEC progenitors, it may as well be speculated that such a transitional phenotype including collagen III synthesis may be characteristic for HCEC progenitor or transient amplifying cells. Therefore, these clonal cell lines may not only represent central and peripheral HCEC, but may also represent differentiated (B4G12) and transitional (H9C1) cells of the corneal endothelium.

At an experimental approach, CEC transplantation is investigated at an in vitro level [Alvarado et al., 1981; Bednarz et al., 1996; Abochamat et al., 1999; Bohnke et al., 1999; Engelmann et al., 1999; Chen et al., 2001] and in animal studies [Jumblatt et al., 1978; Gospodarowicz et al., 1979a, b; McCulley et al., 1980; Joo et al., 2000; Ishino et al., 2004; Mimura et al., 2004b; Hsiue et al., 2006] using cell suspensions or engineered cell sheets of cultured CEC. Several groups could demonstrate that cultured HCEC can be transplanted onto denuded donor corneas, where they establish a new functional monolayer [Abochamat et al., 1999; Bohnke et al., 1999; Engelmann et al., 1999; Chen et al., 2001; Amano, 2003; Mimura et al., 2004a]. This method was developed to improve donor corneas that were unsuitable for keratoplasty due to an insufficient endothelial cell density, in order to render such cell-transplanted corneas suitable for such treat-
ment. Recent studies have focused on preparing transplantable CEC sheets that can be used for corneal regenerative applications [Mimura et al., 2004b; Hsiue et al., 2006; Ide et al., 2006; Sumide et al., 2006; Nitschke et al., 2007]. In addition, cell transplantation approaches developed for application in man should be performed under serum-free conditions, because the composition of serum is undefined and its quality can vary greatly. The 2 clonal cell lines described in this work were therefore established in a serum-free culture medium. They express junctional proteins, Na,K-ATPase and 9.3.E-antigen. The staining pattern of junctional proteins and 9.3.E-antigen is comparable to that of primary HCEC, while staining of Na,K-ATPase was weaker in B4G12 cells than in H9C1 cells. We assume that accumulation of Na,K-ATPase at the lateral cell borders in vitro occurs in a time-dependent fashion during cultivation. It can thus be concluded that at least in the case of the B4G12 line the cells are able to form a functionally intact monolayer and could be useful in transplantation experiments under serum-free conditions.

In summary, these clonal cell lines may be useful to study adherence, influence of proliferative processes or metabolic functions of HCEC, and cellular behavior after experimental cell transplantation. Such investigations may also help to gain more knowledge about the differences in peripheral versus central HCEC. However, although our findings led us to hypothesize that the 2 lines represent cell populations derived from different localizations and at different stages of differentiation, further investigations are necessary to corroborate or disprove this hypothesis.

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References


