Alignment and Cell-Matrix Interactions of Human Corneal Endothelial Cells on Nanostructured Collagen Type I Matrices

Rita Gruschwitz,1 Jens Friedrichs,2,5 Monika Valtink,1 Clemens M. Franz,2,4 Daniel J. Müller,2,5,5 Richard H. W. Funk,1,5 and Katrin Engelmann5,6

PURPOSE. To use nanoscopically defined, two-dimensional matrices assembled from aligned collagen type I fibrils as a sheet substratum for in vitro cultivation of human corneal endothelial cells (HCECs). To assess the effect of matrix architecture on HEC morphology and to characterize integrin-mediated HEC-matrix interaction.

METHODS. Cell alignment and cell-matrix interactions of primary HCECs and three different immortalized HCEC populations on native and UV-cross-linked collagen type I matrices were examined by time-lapse microscopy. Specific integrin α2β1 binding to the collagen matrix was demonstrated using a function-blocking α2 antibody. Integrin α2 subunit expression levels of the four HCEC populations were analyzed by Western blot analysis.

RESULTS. All HCEC populations aligned along the oriented collagen fibrils. Primary HCECs and, to a lesser extent, the other tested HCEC populations exerted high traction forces, leading to progressive matrix destruction. Cross-linking of the collagen matrices considerably increased matrix stability. Integrin subunit α2 expression levels of the four cell types correlated with the degree of cell alignment and exertion of traction forces. In turn, blocking integrin subunit α2 reduced cell alignment and prevented matrix destruction.

CONCLUSIONS. HCECs align directionally along parallel arrays of collagen type I fibrils. The interactions of HCECs with collagen type I are primarily mediated by integrin α2β1. Integrin subunit α2 levels correlate with matrix contraction and subsequent destruction. Sustained cultivation of HCECs on ultrathin collagen matrices thus requires matrix cross-linking and moderate integrin α2β1 expression levels. (Invest Ophthal Mol Vis Sci. 2010;51:6303–6310) DOI:10.1167/ioms.105-5368

From the 1Institute of Anatomy, Medical Faculty Carl Gustav Carus, 2Cellular Machines Group, Biotechnology Center, and 3CRTD/DFG-Center for Regenerative Therapies Dresden–Cluster of Excellence, TU Dresden, Dresden, Germany; 4ETH Zürich, Department of Biosystems, Science and Engineering, Basel, Switzerland; 5DFG-Center for Functional Nanostructures, Karlsruhe Institute for Technology, Karlsruhe, Germany; and 6Department of Ophthalmology, Klinikum Chemnitz gGmbH, Chemnitz, Germany.

Supported by a Volkswagen-Stiftung grant (DJM, RHWF).

Submitted for publication February 11, 2010; revised June 24, 2010; accepted June 30, 2010.

Disclosure: R. Gruschwitz, None; J. Friedrichs, None; M. Valtink, None; C.M. Franz, None; D.J. Müller, None; R.H.W. Funk, None; K. Engelmann, None

Corneal endothelial degeneration is the main diagnosis leading to keratoplasty. In recent years, exclusive replacement of the endothelial layer, such as in Descemet membrane endothelial keratoplasty (DMEK) or posterior lamellar keratoplasty (PLK), has come into focus. In addition to using donor corneas for preparing transplantable posterior lamellae, several groups investigated the transplantation of cell sheets composed of in vitro-cultured corneal endothelial cells (CECs) on various natural or synthetic carrier materials (e.g., Descemet’s membrane, coated hydrogel lenses, amniotic membrane, or biodegradable polymers).1–6 Natural carrier materials are considered inhomogeneous concerning their content of deposited biologically active factors and their immunogenic state. Synthetic carriers, though often supplying better mechanical stability, do not offer a biologically enriched microenvironment for cells. Artificial collagen matrices represent a compromise between natural and synthetic matrices. For transplantation purposes, low antigenicity and biodegradability are important advantages of collagen type I matrices. Furthermore, collagen type I carriers were already used to support human CEC (HCEC) growth.7 Although a systematic investigation of the interactions of CEC and matrix carriers was performed using biodegradable, synthetic polymers, such an analysis is missing for collagen type I matrices.6

Recently, a method to create ~3-nm-thick collagen matrices composed of highly aligned collagen type I fibrils was developed. The fibrils display the characteristic 67-nm D-band periodicity typically observed in vivo.8 Various fibroblast cell lines elongate and migrate along the fibrils of the collagen matrices, primarily through the major collagen-binding integrin α2β1.9–11

In this study we characterized the morphology and cell-matrix interactions of four HCEC populations on these oriented collagen matrices for two reasons. First, collagen type I is a defined and inexpensive biopolymer and is, therefore, a promising candidate substratum for CEC sheet production. Second, the nanopatterned collagen type I matrices used in this study are structurally well defined and allow direct visualization of cellular interaction with the matrix fibrils.

MATERIALS AND METHODS

Cell Culture

Media, supplements, and reagents were purchased from Gibco Invitrogen (Karlsruhe, Germany), Sigma Aldrich (Munich, Germany), or Biocrhom AG (Berlin, Germany), unless otherwise stated. Primary HCEC isolation is described elsewhere.12 Primary HCECs and the immortalized HCEC population HCEC-1213,14 were grown in F99HCEC (Ham’s F12/medium 199, 5% FCS, 20 μg/mL ascorbic acid, 10 ng/mL human recombinant FGF-2, 20 μg/mL human recombinant insulin, 2.5 μg/mL amphotericin B, 50 μg/mL gentamicin). The clonal HCEC lines B4,G12...
and H9C1 were grown in serum- and antibiotic-free growth medium (Human Endothelial-SFM) supplemented with 10 ng/mL FGF-2. For passaging, cells were first incubated in enzyme-free cell dissociation buffer for 2 minutes at room temperature (RT; except H9C1), followed by trypsin/EDTA (0.05%/0.02%) for 2 minutes. Detached cells were collected in F99, serum-free protease inhibitor cocktail (cOmplete; 1 tablet/50 mL serum-free medium; Roche Diagnostics, Mannheim, Germany), centrifuged at 100g for 5 minutes and plated at a density of 2000 cells/cm². B4G12 cells were seeded at 4000 cells/cm² because of a slower growth rate and the positive effect of higher cell densities on cell viability. All cultures were grown in T25 tissue culture flasks coated with 10 µg laminin and 10 mg chondroitin sulfate solubilized in 1 mL F99 basal medium for 1 hour at RT. Cells were maintained at 37°C in a humidified atmosphere containing 5% CO₂. Growth medium was changed 5× per week.

Preparation and Cross-Linking of Nanopatterned Collagen Matrices

Ordered collagen type I matrices were prepared on mica surfaces as described previously.8,15 Briefly, mica discs were covered with 80 µL buffer solution (0.2 M KCl, 0.05 M glycine, pH 9.2). Bovine collagen type I solution (Cohesion Inc., Palo Alto, CA) was injected into the buffer solution to a final concentration of 100 µg/mL. After incubation in a humidified atmosphere at RT for 24 hours, the collagen-coated surfaces were washed three times with PBS.

To cross-link collagen matrices, a small volume of 0.5% riboflavin solution was added onto the collagen matrix and exposed to UV-A radiation (370 nm) using a UV-A double diode (Roithner Lasertechnik, Vienna, Austria) at a distance of 1.5 cm (3 mW/cm²) for 25 minutes. Excess riboflavin was removed by several washes with PBS before cell seeding.

Time-Lapse Phase-Contrast Microscopy

Cells were seeded on the collagen type I matrices at a density of 6 × 10³ to 1 × 10⁵ cells/well in 2.5 mL of the respective growth medium. For integrin blocking experiments, cells were pretreated with a monoclonal anti-integrin α5 antibody (10 µg/mL; clone P1H5; Santa Cruz Biotechnology Inc., Santa Cruz, CA). Phase-contrast images were obtained using an inverted microscope (Axiovert 200M; Carl Zeiss, Oberkochen, Germany) with a 10× (NA=0.4) phase-contrast objective (Carl Zeiss) and a charge-coupled device camera (CoolSnap HQ; Photometrics, Munich, Germany), driven by imaging software (Metamorph; Universal Imaging, Sunnyvale, CA). Cells were maintained at 37°C in a humidified atmosphere containing 5% CO₂. Images were collected every 3 minutes for a total duration of 20 hours. For each cell type and experimental condition, at least three movies were recorded and analyzed.

Scanning Electron Microscopy

HCEC-12 cells were grown on the nanostructured collagen type I matrix for 18 hours. By this time, matrix disruption could be observed. Cells were fixed in 2.5% glutaraldehyde overnight and subsequently washed in 0.2 M cacodylate buffer. Samples were dehydrated in ascending ethanol concentrations (30%–100% in six steps), critically point-dried, and sputtered with gold particles. Samples were analyzed in a cold field emission scanning electron microscope (JSM 7500F; JEOL Germany GmbH, Eching, Germany).

Cell Polarization and Shape Index

Cell polarization and morphology were determined from phase-contrast images collected 150 minutes after cell seeding. Cell contours were outlined manually (Photoshop 7.0; Adobe, San Jose, CA), and geometrical features were calculated (Illustrator 10.05 [Adobe] and MATLAB 7 [MathWorks, Natick, MA]). An ellipse was fitted to the cell outline to determine its major axis orientation relative to the positive x-axis in the image frame (δ in degree). Collagen orientation was determined from 10 representative aligned cells for each frame.14 The average deviation δ of the major cell axis from collagen fibril orientation was calculated for all cells in a frame, and cell orientation was quantified and compared between different cell populations. The best-suited quadrant was chosen so that the maximum angle difference was 90°, and cells were considered to have aligned along the collagen fibrils if δ fell within ±15° of the angle of the collagen orientation. To evaluate cell elongation, the shape index was obtained for each cell by determining the relation between the minor and the major axis of the ellipse that was fitted to the cell contour. Values varied between 0 and 1, with values approaching 0 representing a highly elongated cell and values approaching 1 representing a spherical cell. Cells with a shape index >0.8 were excluded from orientation analysis. A two-tailed Mann-Whitney test was used for statistical analysis.

Western Blot Analysis

Cells were grown to confluence and lysed with ice-cold lysis buffer (1% Triton X-100, 150 mM NaCl, 10 mM Tris/HCl pH 7.5, 1 mM EDTA, 25 mM NaF, 1 tablet/10 mL protease inhibitor cocktail; cOmplete Mini; Roche Diagnostics). Lysates were centrifuged at 16,000g, and the soluble fractions were either used immediately or stored at −20°C. Concentration of soluble protein was determined using the Bradford

Downloaded From: https://iovs.arvojournals.org/pdfaccess.ashx?url=/data/journals/iovs/932969/ on 08/09/2018
protein assay (Bio-Rad, Hercules, CA). Samples were diluted to equal concentration with lysis buffer, separated on 6% SDS-polyacrylamide gels, and transferred onto nitrocellulose membranes by semidry Western blotting at 100 V for 1 hour. Subsequently, membranes were blocked in 5% nonfat dry milk/PBS-T and probed with a primary monoclonal mouse anti-human integrin α2 subunit antibody (clone 2; BD Transduction Laboratories, Lexington, KY) at a dilution of 1:500 for 1 hour at RT and a secondary polyclonal sheep anti-mouse IgG HRP-conjugated antibody at 1:3300 for 1 hour at RT. Protein bands were visualized using ECL chemiluminescence detection and digital image recording. Probing for GAPDH (mouse monoclonal, clone 6C5; Abcam, Cambridge, UK; dilution 1:20,000, incubation overnight at 4°C) served as loading control.

RESULTS

Preparation of Nanopatterned Collagen Type I Matrices

The applied protocol yields collagen type I matrices composed of a 3-nm thin monolayer of highly aligned collagen fibrils. The matrix was characterized by atomic force microscopy (Fig. 1). Matrix formation requires injection of the collagen solution into an appropriate buffer solution, which facilitates self-assembly of the collagen monomers into parallel fibers. Alignment of collagen type I fibrils is achieved by an intrinsic yet unexplained property of the mica substrate. As shown in previous studies,8,9,15,17,18 the matrices form with extremely high precision and reproducibility. Under defined buffer conditions, variabilities in the collagen alignment between matrices can be neglected.

Cell Populations

Four morphologically different HCEC populations were used in this study. Primary HCECs from human donor eyes were of an initially epithelioid, in later passages more fibroblastoid, morphology (Fig. 2A). The immortalized population HCEC-1213,14 was derived from whole corneal endothelium and, as such, consisted of several subpopulations. It is composed of small polygonal cells and larger elongated cells (Fig. 2B). Two clonal daughter cell lines derived from HCEC-12 by repeated cloning...
from a breaking edge in the mica support, which leads to a different orientation of the collagen matrix. Scale bar, 100 μm. (B) Magnification of the inset in (A). Scale bar, 10 μm. Parallel, aligned collagen type I fibrils and collagen-free areas are visible between cells. At the cell edges, collagen fibrils are bundled and ripped from the mica surface. (C) Magnification of the inset in (B). Scale bar, 3 μm. Bundled collagen type I fibrils can be seen beside and above a cell that has migrated underneath the nanostructured collagen matrix. Aligned fibrils are visible next to the cell and collagen-free areas.

Polarization and Migration on Nanostructured Collagen Matrices

Polarization and migratory response of the different HCEC populations on native and cross-linked collagen matrices was characterized by time-lapse phase-contrast microscopy over a period of 20 hours. Primary HCECs immediately adhered to native collagen matrices and aligned along the direction of the collagen fibrils within the first 30 minutes after seeding (Fig. 3A). After approximately 75 minutes, fibrillar structures became visible as fine dark lines along the main axis of the cells. These structures represented structural defects introduced into the collagen matrix resulting from cellular traction forces. After approximately 2 hours, the matrix was extensively degraded, resulting in partial clumping of cells and matrix. Within 2 to 6 hours, the matrix was completely destroyed (Fig. 3A, Movie S1; all Movies are available at http://www.iovs.org/content/51/12/6303/suppl/DC1). In contrast, cross-linked matrices supported primary HCEC alignment for extended periods of time (Fig. 3B). As with native matrices, cell attachment and alignment occurred within 15 minutes. Time-lapse phase-contrast images demonstrated a predominant migration of the cells along the fibrils in both directions, indicating that the cells used the collagen fibrils as tracks to travel considerable distances of up to 500 μm (Movie S2). After 5 to 10 hours, cells started either to bundle collagen fibrils or to slowly peel the collagen from the mica support (Fig. 3B).

HCEC-12 started to spread on native collagen matrices approximately 15 to 45 minutes after seeding (Fig. 4A, Movie S3), and a few cells started to move non-directionally. After 2.5 hours, most of the cells had aligned and the first fibrillar matrix structures became visible. Cell matrix aggregation occurred between 5 and 15 hours after seeding and resulted in matrix disruption. Scanning electron microscopic analysis confirmed matrix disruption by the cells (Fig. 5). On cross-linked collagen matrices (Fig. 4B, Movie S4), cells spread within 30 to 50 minutes and aligned after approximately 2.5 hours. The first structural defects in the matrix became visible after 5 hours. The collagen matrix started to tear off after 7 to 10 hours and ultimately detached, together with the cells from the mica. Alignment of this population was, therefore, similar on native and cross-linked collagen but was delayed compared with primary HCECs.

On native collagen (Fig. 6A, Movie S5), B4G12 cells adhered within 45 minutes but retained a roughly spherical shape for up to 2 hours. Thereafter, most of the cells stretched into an elliptical shape along the collagen fibrils. After 10 to 18 hours, fibrillar structures parallel to the collagen fibril direction became visible. In contrast to primary HCECs, the collagen matrix was never completely destroyed by B4G12 cells. Cell behavior
on cross-linked collagen matrices was similar to that on native matrices (Fig. 6B, Movie S6), but the cross-linked matrix remained intact throughout the entire observation period. Compared with primary HCECs and HCEC-12, migration of B4G12 cells on the matrices was markedly limited.

H9C1 cells attached to native collagen (Fig. 7A, Movie S7) within 30 minutes, and most cells aligned parallel to the collagen fibers within 2 hours. In contrast to B4G12 cells, H9C1 cells were highly elongated and often featured several extensions. Compared with primary HCECs and HCEC-12 cells, the migration of H9C1 cells was reduced. After approximately 5 hours, bundling of collagen fibrils became visible and sometimes culminated in cell matrix aggregation and complete matrix destruction. Cross-linking prevented overall matrix destruction but did not always prevent local matrix disruption (Fig. 7B, Movie S8).

Cell Orientation and Shape Index

Cell orientation and shape index of cells on native and cross-linked collagen matrices were determined from images collected 150 minutes after seeding. Cell orientation was analyzed for cells with a shape index less than 0.8. As depicted in Figure 8A, most cells of each population displayed clear alignment with the collagen fibrils, albeit to different degrees. Cell alignment of primary HCECs could only be determined on cross-linked matrices, where 64.6% of the cells fell within $\delta = 15^\circ$. Hence, primary HCECs showed better orientation along the collagen fibrils than HCEC-12 on cross-linked matrices (54.3%) but aligned to a lesser degree than B4G12 (79.0% on cross-linked, 68.7% on native matrices) or H9C1 (70.9% on cross-linked, 59.2% on native matrices) cells. Of all four populations, HCEC-12 displayed the least degree of alignment on native (54.9%) and on cross-linked matrices, whereas B4G12 cells showed the best orientation on native and on cross-linked matrices.

The ratio between the major and the minor axes of an ellipse fitted to the outline of a cell was used as a measure of cell elongation, with a value approaching 0 indicating a highly elongated cell and a value approaching 1 denoting a rounded cell. Of the examined populations, primary HCECs showed the highest degree of elongation (Fig. 8B), with 74.2% of the cells having a shape index $\leq 0.5$. Similarly, H9C1 cells were well elongated, with 73.6% of the cells having a shape index $\leq 0.5$ on native matrices and 61.1% on cross-linked matrices. HCEC-12 cells were less elongated, with 49.5% of all cells having a shape index $\leq 0.5$ on native matrices and 52.7% on cross-linked matrices. B4G12 cells showed the least degree of elongation, with 32.2% of all cells having shape indices $\leq 0.5$ on native matrices and 46.7% on cross-linked matrices. Elongation of HCEC-12 and H9C1 cells was higher on native than on cross-linked membranes, whereas B4G12 cells elongated slightly better on cross-linked collagen.

Protein Levels of Integrin Subunit $\alpha_2$

Cellular traction forces transmitted by the integrin $\alpha_2\beta_1$ receptor are important for cell alignment along collagen fibrils. 9

![Figure 7](https://iovs.arvojournals.org/pdfaccess.ashx?url=/data/journals/iovs/932969/)  
**Figure 7.** Time-lapse sequences of the clonal line HCEC-H9C1 on ordered collagen type I matrices. (A) H9C1 cells begin to align after 30 minutes. After 5 hours, bundled collagen fibers become visible, and sometimes cell matrix aggregates are visible by the end of the observation period. (B) On cross-linked collagen type I, H9C1 cells adhere within 30 minutes and align within the first 2 hours. Local matrix disruptions become visible by 5 hours, but the matrix is not destroyed completely. White arrow: direction of collagen fibrils. Small black arrows: bundled collagen fibers. White arrowhead: cell aggregates after matrix disruption. Scale bar, 100 $\mu$m.

![Figure 8](https://iovs.arvojournals.org/pdfaccess.ashx?url=/data/journals/iovs/932969/)  
**Figure 8.** Box-whisker plots of cell orientation (A) and shape index (B) on native and UV-A/riboflavin cross-linked matrices. Box-whisker plots present 50% of the data points within the box and 80% within the whiskers. The black line represents the median and the white line represents the mean of all data points. Cells with a shape index $>0.8$ were excluded from orientation analysis. Numbers of examined cells are given in angled brackets. ns, $P > 0.05$; *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$.  

Downloaded From: https://iovs.arvojournals.org/pdfaccess.ashx?url=/data/journals/iovs/932969/ on 08/09/2018
Given that the integrin α2 subunit exclusively associates with the integrin β1 subunit, determining α2 subunit levels allows evaluation of the total amount of integrin α2β1 receptor. To assess whether protein levels of integrin α2β1 correlate with the degree of cell-matrix interactions, α2 levels of the four cell populations were determined by Western blot analysis. Primary HCECs had the highest integrin subunit α2 levels, whereas HCEC-12 and H9C1 cells produced markedly lower amounts of the integrin subunit α2. Integrin subunit α2 levels of B4G12 were almost undetectable (Fig. 9). These findings correlate with the observation that primary HCECs attached faster to the collagen matrix and exerted markedly higher traction forces than the other three examined HCEC populations. The fact that integrin subunit α2 can hardly be detected in B4G12 cells reflects their limited capacity to develop traction forces on the collagen matrices. Surprisingly, the ability to align on the collagen matrices is not correlated with the amount of integrin α2β1.

**Blocking of Integrin Subunit α2**

Specific cell binding to the collagen type I matrices was demonstrated using a function-blocking antibody to the integrin α2 subunit. Antibody-treated primary HCECs could still adhere to the matrix but were unable to align and migrate along the collagen fibrils. Most of the cells remained round within the observation period of 20 hours. In contrast to untreated primary HCECs, antibody-treated cells did not destroy the native matrix (Fig. 10). Antibody-treated cells of the immortalized population HCEC-12 were able to adhere to the collagen fibrils and aligned within 15 minutes. In contrast to untreated HCEC-12, matrix destruction and cell clumping could not be observed over the entire observation period (Fig. 10). B4G12 cells pretreated with the antibody started to align along the collagen fibrils after approximately 2.5 hours. Alignment was less pronounced compared with the parental population HCEC-12, but B4G12 cells were still able to remodel the matrix (Fig. 10). Similar to primary HCECs, antibody-treated H9C1 cells adhered to the matrix after 15 minutes and started to elongate after approximately 2.5 hours. However, cell elongation was randomly directed (Fig. 10). Furthermore, H9C1 cells did not remodel or destroy the matrix. These results indicate that matrix destruction was impaired by the integrin subunit α2 blocking antibody, whereas cell alignment was partially independent of integrin α2 blockage.

---

**Figure 9.** Western blot analysis of integrin subunit α2 production in HCEC populations. Equal amounts of total protein were loaded in each lane, as confirmed by staining for GAPDH (loading control). Primary HCECs gave a markedly stronger band for integrin subunit α2 than the other three HCEC populations. In B4G12 cells, integrin subunit α2 was almost not detectable.

**Figure 10.** Time-lapse video microscopy of primary HCECs, immortalized HCEC-12, and clonal HCEC lines B4G12 and H9C1 after pretreatment with a monoclonal anti-integrin α2 antibody. Primary HCECs attached but did not align along the collagen fibrils. HCEC-12 showed strong alignment along the collagen fibrils but, unlike their untreated counterparts, did not destroy the matrix within 20 hours. B4G12 cells attached to the matrix and started to align and remodel the matrix after 2.5 hours. Bundled collagen fibers became visible at 20 hours of observation time (small black arrows). H9C1 cells attached to the matrix but showed no alignment. Elongation of the cells was rather random, and the matrix remained intact over 20 hours. **White arrows:** orientation of the collagen fibrils. Scale bar, 100 μm.
**Discussion**

The aim of this study was to investigate the specific interaction of four different HCEC populations with ordered arrays of collagen type I fibrils and to examine whether these nanostructured collagen matrices may serve as a substrate for HCEC cultivation. It was previously shown that nanotopographic cues can influence the behavior of ocular cells. For example, SV40-transfected human corneal epithelial cells elongated and migrated along pitches of 400- to 2000-nm width on polyurethane surfaces. Other experiments using silicon surfaces with pitches revealed that cells do not respond to nanotopographic cues when the distance between the structures is below a minimum length scale. This phenomenon was termed contact acuity. Such behavior in response to nanotopographic cues has also been described for keratocytes, fibroblasts, and myofibroblasts. Interestingly, contact acuity was determined to be specific for each cell type and was also dependent on microenvironmental conditions. To our best knowledge, a study characterizing the response of HCECs to nanotopographic cues has not yet been conducted.

The immortalized HCEC population HCEC-1213 was chosen because it allowed us to perform multiple and repetitive experiments with cells of one donor without changes in its phenotype. The two clonal daughter cell lines B4G12 and H9C1, which originate from the HCEC-12 population, were chosen because they represent subpopulations derived from different localizations within the cornea (center, periphery) and show distinct differences in their phenotype. With respect to future clinical implementation of HCEC sheet design, we also chose to examine primary cultures of HCECs that were isolated from different donor corneas. We observed that the examined HCEC populations coaligned with fibrils of the underlying collagen matrix, suggesting that HCECs specifically interact with collagen type I. However, the cell populations showed differences in the extent of elongation, morphology, and migration on the collagen type I matrices. This demonstrated that in addition to the known interindividual differences between cells isolated from different donors, subpopulations of corneal endothelium from a single cornea can show marked variations in key features such as adhesion and integrin expression. These differences can have a major impact on the outcome of studies that focus on developing new techniques that might be implemented in clinical use. These techniques include the generation of cell matrix sheets for endothelial keratoplasty. Hence, the choice of the model (cell population) that will be used in such preclinical studies is of utmost importance.

The examined HCEC populations also varied in the extent to which they disrupted the collagen substrate. Cell elongation and collagen fiber formation are likely the result of the bundling of collagen fibrils at the leading edge of the cell, with simultaneous disruption of the collagen network along the side of the cell. A similar alignment mechanism was recently demonstrated for human osteosarcoma cells using life-cell time-lapse atomic force microscopy imaging. The bundling of fibrils increases traction forces in the direction of the fibrils and in this way promotes cell elongation and directional migration. We could previously show that elongation and directional migration of the osteosarcoma cells on collagen type I involved integrin-mediated adhesion. A similar mechanism appears to be involved in the response of HCECs on the collagen matrix because the degree of matrix destruction correlated with integrin subunit α2 protein levels. However, it appears that the integrin subunit α2 is primarily responsible for matrix deformation and, to a lesser extent, for cell alignment. This also implies that integrins other than integrin αβ might be involved in the interaction of corneal endothelial cells and collagen type I.

Importantly, it could be demonstrated that matrix stability could be increased by cross-linking using riboflavin under UV-A irradiation. This method is based on the formation of radicals mediated by irradiated riboflavin that react with collagen side chains, thereby establishing covalent bonds between adjacent collagen molecules. In contrast to other cross-linking methods, such as glutaraldehyde treatment, no additional hydrocarbon moieties are introduced. For all cell subtypes tested, cross-linking delayed or even prevented destruction of the matrix during the observation period of 20 hours. In this context, it is interesting to note that UV-induced cross-linking did not interfere with cell alignment. This is in contrast to glutaraldehyde-based cross-linking, which prevented the alignment of osteosarcoma cells on patterned collagen matrices. Apparently, riboflavin-based cross-linking retained the ability of the matrix to direct cell alignment and migration, whereas harsher chemical cross-linking procedures did not. Besides strengthening the matrix, cross-linking may also expose additional integrin-binding sites on the collagen fibrils. Jokinen et al. demonstrated that monomeric collagen type I contains three high-affinity sites for the αI domain, whereas mature collagen fibrils display reduced αI domain binding, possibly as a result of masked integrin binding sites during fibrillogenesis. Riboflavin-UV-based cross-linking is known to increase the diameter of collagen fibrils in the cornea, but the precise ultrastructural effects are unknown. It remains to be clarified whether cross-linking affects the accessibility of high-affinity integrin binding sites.

We found that nanostructured collagen type I matrices may serve as potential model systems to further investigate the early steps of cell-matrix interaction. Although native matrices seemed unsuitable for long-term cultivation of HCECs, matrix stabilization could allow the use of ordered collagen structures as an HCEC substratum. We demonstrated the general suitability of photochemically stabilized collagen type I matrices for the cultivation of HCECs. However, it remains to be tested whether nanostructured collagen matrices can be used as substrates for the production of transferable or transplantable HCEC sheets and whether the directed growth of HCECs is a desirable feature of a cell sheet transplant. Notably, if the matrix were to be used for transplantation or cell sheet transfer, it would require further stabilization to be resistant to cellular traction forces and to prevent dislocation of the matrix from the supporting material during the cultivation phase. Furthermore, because the formation of nanopatterned collagen type I matrices requires mica as a supporting material, a technique that allows lifting of the matrix from this supporting material without destroying it must first be developed. If these technical obstacles can be overcome, it is possible that, for example, a combination of thermoresponsive polymers and nanostructured matrices may offer a great potential toward cell sheet design in the future. The use of thermoresponsive polymer substrates has already been described for generating HCEC sheets and transplantable sheets of corneal epithelial cells. Until then, nanostructured collagen type I matrices may serve as potential model systems to further investigate the early steps of cell-matrix interaction (e.g., corneal epithelial cells).

**References**


Downloaded From: https://iovs.arvojournals.org/pdfaccess.ashx?url=/data/journals/iovs/932969/ on 08/09/2018


