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Characterization of Corneal Keratocyte Morphology and Mechanical Activity within 3-D Collagen Matrices

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Abstract

The purpose of this study was to assess quantitatively the differences in morphology, cytoskeletal organization and mechanical behavior between quiescent corneal keratocytes and activated fibroblasts in a 3-D culture model. Primary cultures of rabbit corneal keratocytes and fibroblasts were plated inside type I collagen matrices in serum-free media or 10% FBS, and allowed to spread for 1–5 days. Following F-actin labeling using phalloidin, and immunolabeling of tubulin, α -smooth muscle actin or connexin 43, fluorescent and reflected light (for collagen fibrils) 3-D optical section images were acquired using laser confocal microscopy. In other experiments, dynamic imaging was performed using differential interference contrast microscopy, and finite element modeling was used to map ECM deformations.

Corneal keratocytes developed a stellate morphology with numerous cell processes that ran a tortuous path between and along collagen fibrils without any apparent impact on their alignment. Fibroblasts on the other hand, had a more bipolar morphology with pseudopodial processes ($p \leq 0.001$). Time lapse imaging of keratocytes revealed occasional extension and retraction of dendritic processes with only transient displacements of collagen fibrils, whereas fibroblasts exerted stronger myosin II-dependent contractile forces ($P < 0.01$), causing increased compaction and alignment of collagen at the ends of the pseudopodia ($P < 0.001$). At high cell density, both keratocytes and fibroblasts appeared to form a 3-D network connected via gap junctions. Overall, this experimental model provides a unique platform for quantitative investigation of the morphological, cytoskeletal and contractile behavior of corneal keratocytes (i.e. their mechanical phenotype) in a 3-D microenvironment.

Keywords

Cornea; Wound Healing; Keratocyte; Collagen matrix; Cell mechanics; Cytoskeleton

INTRODUCTION

Corneal keratocytes are the primary cells populating the corneal stroma. They are normally considered quiescent *in vivo*, with a stellate morphology and a cortical distribution of f-actin (Jester et al., 1994). Following corneal injury or surgery, keratocytes become activated and transform into fibroblasts or myofibroblasts. These transformed keratocytes repopulate the

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wound space and are thought to generate the forces required for wound contracture and ECM remodeling (Jester and Chang, 2003). Culture of keratocytes in 2-D culture under serum-free conditions maintains the quiescent phenotype normally observed in vivo (Beales et al., 1999; Jester et al., 1994). Exposure to serum results in fibroblast differentiation, as indicated by the assumption of a bipolar morphology, formation of intracellular stress fibers, and an altered proteoglycan expression profile (Dahl, 1981; Hassell et al., 1992). While much is known regarding the biochemical and morphological characteristics of corneal keratocytes and fibroblasts, much less is known about how the *mechanical* behavior of these cells is regulated. Furthermore, previous studies have been performed primarily using 2-D substrates, and keratocytes reside within a complex 3-D extracellular matrix in vivo. Significant differences in cell morphology, adhesion organization, and mechanical behavior have been identified between 2-D and 3-D culture models, with 3-D models more closely mimicking in vivo cell behavior (Abbott, 2003; Bard and Hay, 1975; Cukierman et al., 2001; Cukierman et al., 2002; Doane and Birk, 1991; Doyle et al., 2009; Friedl and Brocker, 2000; Grinnell et al., 2003; Rhee et al., 2007; Tomasek et al., 1982). Furthermore, unlike rigid 2-D substrates, 3-D models also allow investigation of cell mechanical activity and cell-induced matrix remodeling - key events in the wound healing process.

We have previously established a model for dynamic investigation of cell-matrix mechanical interactions, by plating fibroblasts inside 3-D fibrillar collagen matrices, and performing high magnification time-lapse DIC imaging (Petroll and Ma, 2003; Petroll et al., 2008b; Vishwanath et al., 2003). In addition, we have developed quantitative confocal imaging techniques to assess 3-D cell morphology, f-actin organization, and both the pattern and amount of local cell-induced collagen matrix reorganization (Kim et al., 2006; Lakshman et al., 2007). The combination of these two techniques allows assessment of both rapid changes in cellular contractility (which can occur within minutes), and more gradual changes in cell-induced compaction and alignment of ECM (which can occur over several days). While these approaches have been used to study the regulation of fibroblast behavior in 3-D collagen matrices (Kim et al., 2006; Petroll and Ma, 2008), they have not yet been applied to quiescent corneal keratocytes.

In this study, we combine these techniques to comprehensively assess and compare the mechanical phenotypes of both corneal keratocytes and corneal fibroblasts in 3-D culture. Our data demonstrates that in contrast to corneal fibroblasts, which develop a bipolar morphology and generate sustained contractile forces on the ECM (contractile mechanical phenotype); corneal keratocytes assume a stellate shape and exert much less force (quiescent mechanical phenotype). Nonetheless, keratocytes are able to extend and retract dendritic process and induce small, transient displacement of collagen fibrils, potentially allowing them to survey their environment without permanently altering the ECM. At high density, both keratocytes and fibroblasts appear to interconnect via gap junctions, forming a network similar to that observed in vivo. Overall, this experimental model should provide a unique platform for quantitative investigation of the morphological, cytoskeletal and mechanical responses of corneal keratocytes to growth factors and other stimuli in a 3-D microenvironment.

MATERIALS AND METHODS

Cells

Corneal keratocytes were isolated from rabbit eyes obtained from Pel Freez (Rogers, AR, USA) as previously described (Petroll et al., 2003). Cells were cultured in flasks with either: (1) keratocyte medium (Jester and Chang, 2003) consisting of Dulbecco's modified Eagle's minimum essential medium (DMEM; Invitrogen, Carlsbad, CA), supplemented with 1% RPMI vitamin mix (Sigma-Aldrich, Saint Louis, MO), 100 μ M nonessential amino acids (Invitrogen, Carlsbad, CA), 100 μ g/ml ascorbic acid, and 1% penicillin/streptomycin amphotericin B

(Fungizone; BioWhittaker, Inc., Walkersville, MD) without serum; or, (2) fibroblast medium consisting of DMEM supplemented with 1% penicillin/streptomycin amphotericin B plus 10% fetal calf serum (FBS; Sigma-Aldrich, Saint Louis, MO).

Collagen Matrices

Hydrated collagen matrices were prepared by mixing neutralized bovine dermal collagen (Purecol; Inamed, Fremont, CA) with 10X DMEM to achieve a final collagen concentration of 2.48 mg/ml. A 50 μ l of suspension of cells was then mixed with 500 μ l of collagen solution. After adjusting the pH to 7.2 by addition of 0.1N NaOH, the cell/collagen mixture was preincubated at 37°C for 4 minutes, and 30- μ l aliquots were then spread over a central 12mm diameter circular region on Bioptechs culture dishes (Delta T; Bioptechs, Inc., Butler, PA). The dishes were then placed in a humidified incubator for 60 minutes for polymerization and overlaid with 1.5 ml of keratocyte media (serum-free), fibroblast media (10% FBS). In some experiments, the Rho-kinase inhibitor Y-27632 (10 μ M or 50 μ M) was added to the media at the time of plating the 3-D matrices. Matrices with both low cell density (2×10^3 cells/matrix) and high cell density (5×10^4 cells/matrix) were used.

Static Imaging

After 1–5 days of 3-D culture, cells were fixed using 3% paraformaldehyde in phosphate buffer for 15 min and permeabilized with 0.5% Triton X-100 in phosphate buffer for 3 min. To label f-actin, Alexa Fluor 488 phalloidin was used (1:20, Invitrogen). In some experiments, cells were double labeled by incubating with Cy3-conjugated monoclonal anti- β -tubulin (1:100, Sigma) for 60 minutes. In other experiments immunolabeling with connexin 43 or α -smooth muscle actin (α -SM actin) was performed. Following incubation in 1% BSA for 60 minutes to block non-specific binding, cells were incubated for 1 hour in mouse monoclonal antibodies to either human connexin 43 (1:100, Millipore, Billerica, MA) or α -SM actin (1:100, Sigma-Aldrich, St. Louis, MO) in 1% BSA. Cells were then washed in buffer and incubated for 1 hour in affinity-purified Rhodamine conjugated goat anti-mouse IgG (1:20, MP Biomedicals, Solon, OH).

Constructs were imaged using laser scanning confocal microscopy (leica SP2, Heidelberg, Germany). A HeNe laser (633 nm) was used for reflection imaging, and Argon (488 nm) and GreNe (543 nm) lasers were used for fluorescent imaging. Stacks of optical sections (z series) were acquired using a 63 $\times$ water immersion objective (1.2 NA, 220 μ m free working distance) and 20 $\times$ objective (0.7 NA, 590 μ m free working distance). Changes in cell morphology were measured using MetaMorph (Molecular Devices, Sunnyvale, CA). Sequential scanning was used to image double-labeled samples to prevent cross-talk between fluorophores.

Dynamic Imaging

A Nikon TE300 inverted microscope (TE300; Nikon, Tokyo, Japan) was used for time-lapse DIC imaging. For all experiments, the stage, shutters and camera hardware were controlled using a PC running MetaMorph as previously described (Petroll et al., 2003). Most experiments used a Bioptechs Delta T microincubation system and objective heater to maintain temperature at 37°C. In these experiments, dishes were continuously perfused while on the microscope with media containing HEPES buffer at a rate of 6ml/hr; note that open dish perfusion was used to minimize flow through the matrices (Petroll and Ma, 2003; Petroll et al., 2008a). Cells were first allowed to acclimate to the microscope microincubation system for 1 hour. Time-lapse imaging was then performed at 1–2 minute intervals using a 20X dry objective with Nomarski DIC. Three dimensional data sets were obtained at each time point by repeating the acquisition at five to six sequential focal planes in z steps of 2–3 μ m. In some experiments, after 60 min of time-lapse imaging, perfusion was stopped and cytochalasin D (final concentration 25 μ M) was added to assess the effect of f-actin on the cell-induced matrix deformation. In other

experiments, the myosin II ATPase inhibitor blebbistatin (final concentration 100 μ M, Toronto Research Chemicals, Downsview, Canada) was used to assess the role of myosin II in mediating the cell matrix interaction. This concentration provides maximal inhibition of non-muscle myosin IIs, without affecting unconventional myosins (Allingham et al., 2005; Straight et al., 2003). Because blebbistatin has a phototoxic effect with concomitant loss of pharmacological activity at wavelengths below 500 nm, a 570 nm long pass filter was inserted into the light path (E570LP, Chroma Technology Corp, Rockingham, VT).

Additional experiments were performed to assess the effects of nocodazole (which disrupts microtubules), on keratocyte mechanical behavior. These experiments were performed using a humidified environmental chamber maintained at 37°C and 5% CO₂ (In Vivo Scientific, St. Louis, MO). Thus, these experiments did not require the use of HEPES buffer or perfusion. Following a 1 hour acclimation period and 60 minutes of time-lapse imaging in basal media, nocodazole was added (final concentration 10 μ M); imaging was then continued for an additional 2 hours.

Cell Morphology

Changes in cell morphology were measured using MetaMorph as previously described (Kim et al., 2006). Projected cell length and breadth was calculated by outlining the maximum intensity projection image of a cell (generated from the f-actin z-series), thresholding, and applying the Integrated Morphometry Analysis (IMA) routine. The length is calculated by IMA as the span of the longest chord through the object; the breadth is the caliper width of the object, perpendicular to the longest chord. The height of cells was calculated by measuring the distance between the first and last planes in the z-series in which a portion of the cell was visible.

Global Matrix Remodeling

DIC imaging was used to measure global matrix contraction. Since the bottom of the matrices remains attached to the dish, cell-induced contraction results in a decrease in matrix height (Grinnell, 2000). Height was measured by focusing on the top and bottom of each matrix at 6 different locations. Measurements were performed in triplicate for each condition, and repeated 3 times. The percentage decrease in matrix height (as compared to control matrices without cells) in both keratocytes and fibroblasts was evaluated.

Assessment of Local Collagen Fibril Orientation

To assess the pattern and amount of local cell-induced matrix reorganization, both the density and alignment of collagen surrounding cells was assessed from reflected light confocal images as previously described (Kim et al., 2006). For each cell, 2–3 sub-regions were assessed both adjacent to the cell body, and at the ends of pseudopodia. The percent of each region occupied by collagen fibrils was then used as an indicator of collagen fibril density (Karamichos et al., 2009; Kim et al., 2006; Lakshman et al., 2007). Using Metamorph, images were thresholded manually to segment out the collagen fibrils, and binarized. The number of segmented pixels was measured from the binary image, and expressed as a percent of the total number of pixels in the original image. Acellular matrices were used as controls.

Fourier transform (FT) analysis was used to assess local collagen fibril alignment as previously described (Kim et al., 2006; Lakshman et al., 2007). This approach is based on the fact that the relative strength of different angle bands within the FT spectrum is an indicator of the relative number and magnitude of fibers oriented at 90° to that angle in the original image. An “orientation index” (OI) was used to quantify the degree of alignment of the collagen fibrils to a cell process (θ_{pseud}) or the cell body ($\theta_{\text{cell body}}$). The OI has a value of 100% for fibers oriented parallel to θ , –100% for an orientation perpendicular to θ , and 0% for a completely random distribution. Acellular matrices were used as controls.

Finite Element Modeling

In order to quantify the cell-induced elastic distortion of the collagen matrix, strain maps were generated using finite element modeling (FEM) as previously described (Kim and Petroll, 2007; Petroll et al., 2004; Vishwanath et al., 2003). Briefly, the FEM was created using ANSYS software by defining nodes coinciding with ECM landmarks from the DIC images at the “relaxed” matrix configuration, which was determined by treating cells with Cytochalasin D. An extra set of fixed boundary nodes was placed in a 2.5 mm circle around the cell. A two-dimensional finite element model was created from the nodes using linear triangular PLANE2 elements, since most of the ECM deformation occurs in the plane of the cell. The matrix is assumed to be linearly elastic and isotropic, with a Young’s modulus of $3.89 \times 10^{-10} \text{ N}/\mu\text{m}^2$, and an effective thickness of 15 μm . These parameters have been determined by simulating our previous force displacement measurements on collagen matrices (made using calibrated microneedles) using a 3-D finite element model (Roy et al., 1997), and further confirmed by measuring the indentation induced by placing disks of known weight on top of the matrices (Dembo and Wang, 1999). A Poisson’s ratio of 0.3 was used (Roeder et al., 2002). To generate a map of ECM deformation, the measured displacements (relative to the relaxed matrix configuration) from time-lapse recordings were applied to the corresponding nodes in the model, and resulting strains induced on the matrix were calculated and displayed.

Statistics

Statistical analyses were performed using SigmaStat version 3.11 (Systat Software Inc., Point Richmond, CA). A t-test or Rank Sum test was used to compare means in experiments with two groups. When there were more than two groups, one way analysis of variance (ANOVA) was used to compare group means, and post-hoc multiple comparisons were performed using the Holm–Sidak method. Differences were considered significant if $P < 0.05$.

RESULTS

Cell Morphology

Consistent with previous observations on 2-D substrates, corneal keratocytes developed a stellate morphology in 3-D matrices (Fig. 1A), with numerous cell processes extending in all directions from the cell body (Fig. 1C) (Jester et al., 1996). Keratocytes had a cortical, membrane associated f-actin organization, with more concentrated labeling near the ends of cell processes (Fig. 2A, arrows). Often these processes had a core of microtubules (Fig. 2B, arrowheads); however, filopodial extensions were also observed in which tubulin was not detected (Fig. 2C, double arrows).

In contrast, corneal fibroblasts cultured in 10% FBS generally developed a bipolar morphology with pseudopodial processes (Fig. 1B). Fibroblasts in 3-D matrices were always oriented nearly parallel to the bottom of the culture dish (Fig. 1D). Within the cell body and pseudopodial processes, arrays of microfilament bundles (stress fibers) were occasionally observed running parallel to the major axis of the cell.

Quantitative analysis of cell morphology confirmed that when compared to fibroblasts, keratocytes were less bipolar, as indicated by an increase in both breadth/length ratio (0.76 ± 0.15 vs. 0.22 ± 0.08 , $P \leq 0.001$) and had fewer extensions in the z-axis, as indicated by decreased projected cell height ($21.8 \pm 10.7 \mu\text{m}$ vs. $11.5 \pm 1.3 \mu\text{m}$, $P \leq 0.01$) (Fig. 1E).

Dynamic Cell Behaviour

To assess the dynamic behavior of cells in 3-D environment, matrices incubated for 24 hours were used. Matrices with low cell density (2×10^3 cells/matrix) were imaged so that the mechanical activity of a single cell could be assessed, minimizing the potential interference

caused by neighboring cells. DIC imaging allowed detailed visualization of the cells and collagen fibrils surrounding them. Time lapse imaging of keratocytes revealed repeated extensions and retractions of dendritic processes, which resulted in small, transient displacements of collagen fibrils (Movie 1). This behavior was observed both with and without perfusion, and thus was not a response to potential flow-induced shear forces on the cells. Cytochalasin D blocked these dynamic events, and resulted in movement of the matrix away from the cell, consistent with a release of cellular tension. In some cases, cytochalasin D had almost no impact on the surrounding matrix, indicating minimal cellular force generation (Fig. 3A & C red tracks). In contrast to keratocytes, fibroblasts exerted significant contractile force on the matrix, as indicated by more pronounced matrix relaxation after disrupting f-actin using cytochalasin D (Fig. 3D, red tracks) and/or blocking myosin II activity using blebbistatin (Movie 2).

FEM (finite element modeling) was used to better visualize and quantify the pattern of cell-induced force generation by analyzing matrix displacement. For this analysis, the ECM configuration after cytochalasin D treatment was used as the “relaxed” or baseline configuration. The FEM strain maps revealed different patterns for fibroblasts and keratocytes. Fibroblasts consistently induced an increase in the matrix tension at the ends of the cell and compression in the middle (Fig. 3F), whereas keratocytes induced less ECM strain in a much more random pattern (Fig. 3E). Since the degree of cell matrix interaction varied from experiment to experiment a total of five experiments were performed for each cell type. Overall the mean maximum principal strain was reduced by half for keratocytes (0.07 ± 0.03) as compared to fibroblasts (0.16 ± 0.07 , $P < 0.01$).

In contrast to cytochalasin D, nocodazole induced retraction of dendritic keratocyte processes and transition to a more circular morphology with thicker, shorter processes surrounding the cell body (Movie 3). Inward displacement of collagen surrounding the cells was observed.

Cell-Induced Matrix Reorganization

3-D confocal images of single cells from low cell density matrices were used to analyze the local collagen matrix remodeling after 24 hours. Fluorescent imaging was used to visualize f-actin and reflected light imaging was used to visualize collagen surrounding cells (Fig. 4). In general, no significant compaction or realignment of collagen fibrils was observed surrounding corneal keratocytes (Fig. 4A, Movie 4). In contrast, collagen at the ends of corneal fibroblasts appeared to be compacted and aligned parallel to the long axis of the cell, and stress fibers (when present) were always aligned parallel to the collagen fibrils (Fig. 4B, Movie 5). Collagen fibrils adjacent to the cell body were either randomly distributed, or compacted and aligned nearly perpendicular to the long axis of the cell. Corneal fibroblasts treated with the Rho kinase inhibitor Y-27632 developed a stellate morphology with long dendritic processes, and there was a marked reduction in local collagen matrix reorganization (Fig. 4C).

To quantify the degree of collagen fibril orientation surrounding individual cells a previously developed FT analysis technique was used (Kim et al., 2006). For each cell, 2–3 sub-regions were taken both adjacent to the cell body and at the ends of pseudopodia. Sub-regions surrounding 6 cells were analyzed under each condition, and matrices without cells were used as controls (for controls θ_{pseud} and $\theta_{\text{cell body}}$ were assumed to be 0°). The OI measurements from the FT analysis are summarized in Fig. 5. Significant alignment of collagen was observed at the ends of pseudopodia for fibroblasts ($31 \pm 5\%$, $P < 0.001$) as compared to keratocytes ($6 \pm 7\%$) and control matrices with no cells ($8 \pm 14\%$). Adjacent to the cell body there was no preferential alignment of collagen under any condition evaluated (OI was not significantly different than 0).

Collagen fibril density was assessed in the image sub-regions surrounding cells, by calculating the area occupied by collagen fibrils (Fig. 6). Collagen density was significantly higher for fibroblasts as compared to keratocytes at the ends of cell processes ($21.3 \pm 4.3\%$ vs. $7.4 \pm 1.4\%$, $P < 0.001$) and along cell body ($11.3 \pm 3.8\%$ vs. $7.4 \pm 1.6\%$, $P < 0.001$) or compared to control matrices without cells ($7.7 \pm 2.0\%$).

Global Collagen Matrix Reorganization

Collagen matrices with or without cells were prepared and incubated for 24 hrs. Global matrix reorganization, which results in matrix contraction, was measured as a decrease in matrix height, using 1hr measurements as a reference. Assessment of global matrix contraction at low cell density showed no increased matrix contraction by keratocytes as compared to controls (no cells) at either 24 hrs or 5 days. Fibroblasts induced increased matrix contraction as compared to keratocytes (Fig. 7) after 24 hrs (25.8 ± 9.2 vs. 5.0 ± 1.7 , $P < 0.001$) and 5 days (39.8 ± 8.2 vs. 8.0 ± 3.0 , $P < 0.01$) of incubation.

Increased Cell Density

At higher cell density, the dendritic process of adjacent keratocytes appeared to interconnect (Fig. 8A, arrows), and connexin 43 was localized to cell-cell junctions (Fig. 8E). Keratocytes were also flatter than in low density cultures (Movie 6), with fewer extensions along the z-axis (Fig. 8C). For fibroblasts, there was an increase in the number of stress fibers as compared to low density cultures (Fig. 8B, Movie 7), and greater matrix compaction (Fig. 8D). Fibroblasts were also interconnected, as confirmed by connexin 43 labeling (Fig. 8F). Approximately 10% of cells showed positive labeling for α -SM-actin, which was localized to the stress fibers (Fig. 9); in contrast, α -SM-actin labeling was negative for fibroblasts plated at low cell density (not shown).

As compared to low cell density cultures, the collagen within the constructs with a higher density of keratocytes appeared somewhat denser and less uniform, suggesting some cell-induced matrix reorganization. Consistent with this result, significant global matrix contraction was produced by both corneal keratocytes and fibroblasts as compared to control matrices without cells (Fig. 10). However, the amount of global matrix contraction was higher for fibroblasts at both time points ($P < 0.001$).

DISCUSSION

In this study, we performed a comprehensive assessment of the morphology, cytoskeletal organization and mechanical behavior of corneal keratocytes and fibroblasts in a 3-D collagen matrix model. Corneal keratocytes maintained in serum-free media and plated inside 3-D matrices exhibited a dendritic/stellate morphology, with numerous cell processes extending in all directions from the cell body. A primarily cortical, membrane associated f-actin localization was observed, and intracellular stress fibers were not detected, similar to corneal keratocytes *in vivo* (Jester et al., 1994; Poole et al., 2003). Keratocyte processes had a core of microtubules with f-actin concentrated near the tips, and often ran a tortuous path between and along collagen fibrils without any measurable impact on their alignment. Dermal fibroblasts form similar "dendritic" cell processes when they spread within unrestrained matrices (Grinnell et al., 2003), or when contractility is blocked by inhibiting myosin II, thus it has been suggested that they are a characteristic feature of cells in a low-tension environment (Rhee et al., 2007).

In contrast, corneal fibroblasts in 3-D collagen matrices had a bipolar morphology with broader pseudopodial processes, consistent with the corneal fibroblast phenotype observed during corneal wound healing *in vivo* (Garana et al., 1992; Moller-Pedersen et al., 1998). Functional differences in cell contractility and the capacity to reorganize extracellular matrix were also

demonstrated. Corneal fibroblasts induced significant global matrix contraction, and collagen fibrils surrounding the cells were compacted and aligned parallel to the long axis of stress fibers and pseudopodia (Kim et al., 2006). Serum contains factors such as lysophosphatidic acid (LPA) and sphingosine-1-phosphate (S1P), which stimulate cell contractility through activation of the Rho/Rho-kinase pathway (Grinnell, 2000; Jiang et al., 2008; Petroll et al., 2008c). Consistent with previous studies using a human corneal fibroblast cell line, blocking Rho kinase reverted rabbit corneal fibroblasts to a more dendritic morphology and induced significantly less collagen matrix reorganization (Kim et al., 2006; Vishwanath et al., 2003). Thus Rho kinase/Myosin II dependent contractility appears to play a key role in mediating the transformation of quiescent *dendritic* corneal keratocytes to an activated fibroblast phenotype.

Using time-lapse DIC imaging, we also investigated the dynamic pattern of force generation by corneal keratocytes and fibroblasts within 3-D collagen matrices. Although corneal keratocytes are generally considered "quiescent", we found that these cells were mechanically active in our 3-D collagen matrix model. Keratocytes repeatedly extended and retracted dendritic processes, which resulted in small, transient displacements of collagen fibrils. Similar low-tension cell-collagen interactions have been observed in corneal fibroblasts following treatment with Y-27632 and/or blebbistatin, and thus appear to be Rho-kinase/myosin II independent (Petroll et al., 2008a). Interestingly, Langerhans cells exhibit rhythmic extensions and retractions of their dendritic processes *in situ*, and it is hypothesized that these movements allow the cells to more efficiently probe their environment for inflammatory cytokines (Ward et al., 2007). Although smaller, such movements may also allow keratocytes to survey their environment, without permanently altering the ECM. Cytochalasin D blocked these dynamic events, and resulted in movement of the matrix away from the cell, consistent with a release of cellular tension.

FEM strain maps revealed different patterns of cellular force generation for fibroblasts and keratocytes. Fibroblasts consistently induced an increase in the matrix tension at the ends of the cell and compression in the middle, whereas keratocytes induced strain in a much more random pattern. Based on the degree of local matrix reorganization and global matrix contraction data, one might expect the mechanical strain produced by fibroblasts to be several fold higher than that of keratocytes; however, it was only twice the magnitude. We suggest that during the 24 hours of incubation of fibroblasts in serum containing media, a significant amount of permanent matrix remodeling occurs (Guidry and Grinnell, 1986). This remodeling would be expected to increase the local stiffness of the ECM surrounding corneal fibroblasts, thereby altering the stress-strain relationship (Tomasek et al., 2002). Thus at 24 hours, much larger forces were likely required by fibroblasts to induce a given amount of measured strain on the matrix.

In contrast to cytochalasin D, nocodazole induced retraction of dendritic keratocyte processes and inward displacement of collagen surrounding the cells. These data are consistent with previous studies demonstrating that microtubules modulate fibroblast contractility by sequestering Rho GEF (Kim and Petroll, 2007; Krendel, 2002; Kwan and Kirschner, 2005; Liu BP, 1998; Ren et al., 1999; Zhang et al., 2001). Keratocytes also transitioned to a more rounded morphology following microtubule disruption, with shorter, thicker cell processes extended at all angles from the cell body. Thus overall, microtubules appear to play a central role in the maintenance of the dendritic cell phenotype in 3-D culture (Grinnell et al., 2003).

It is important to note that while we were able to measure significant strains on the ECM using DIC time-lapse imaging, we did not detect significant local or global collagen matrix reorganization by corneal keratocytes plated at low density, as compared to control matrices without cells. Apparently, at low cell density, the small local strains generated by keratocytes are not sufficient to cause measurable global matrix contraction. Furthermore, the local

alignment and density of collagen is somewhat variable, which reduces the signal to noise ratio (and thus the sensitivity) of these outcome measures. In order to increase the amount of signal, and to assess the cell-cell interactions which occur *in vivo*, 3-D culture models with a higher cell density were also evaluated. The collagen within these constructs was denser and less uniform than controls, suggesting some keratocyte-induced matrix reorganization. Furthermore, small, but statistically significant global matrix contraction was measured after both 24 hours and 5 days of incubation. Our results differ from a previous report in which corneal keratocytes plated at a similar density did not induce significant global matrix contraction as compared to acellular controls; however, a floating matrix contraction assay was used in this study (Jester and Chang, 2003). Overall, our data indicate that although fibroblasts induce larger forces and much more significant matrix reorganization, keratocytes are not completely devoid of mechanical activity.

In high cell density fibroblast cultures, global matrix contraction was significantly increased as compared to low density cultures. In addition, there was a striking increase in the number intracellular stress fibers, and the intensity of f-actin labeling. The formation of focal contacts and stress fibers by fibroblasts are tension-dependent processes (Burridge and Chrzanowska-Wodnicka, 1996; Rivelino et al., 2001; Tamariz and Grinnell, 2002), thus the increase in stress fibers likely reflects an overall increase in cellular tension. Consistent with our results, Grinnell and coworkers previously demonstrated that dermal fibroblasts plated at high density within collagen matrices develop a bipolar shape and form focal adhesions within 1 hour (Tamariz and Grinnell, 2002); whereas cells plated at low density have a stellate morphology and no detectable focal adhesions even after 4 hours. Thus it is likely that at high cell density, rapid remodeling increases the mechanical stiffness of the matrices, which better supports and upregulates development of stress fibers within corneal fibroblasts. Interestingly, approximately 10% of these cells showed μ -SM-actin labeling of stress fibers, consistent with previous results using rigid substrates (Jester et al., 1996).

At higher cell density, f-actin labeling indicated that both keratocytes and fibroblasts formed an interconnected network within the 3-D matrices. Keratocytes also appeared flatter than observed in low density cultures, with fewer extensions along the z-axis; similar to their organization *in vivo* (Jester et al., 1994; Poole et al., 2003). Both keratocytes and fibroblasts had positive connexin 43 labeling corresponding to apparent sites of cell to cell interactions. Functional gap junctions containing Connexin 43 have been shown to interconnect keratocytes within the normal cornea, as well as fibroblasts within corneal wounds (Jester et al., 1994; Jester et al., 1995; Nishida et al., 1988; Watsky, 1995), and intercellular communication between gap junctions may play an important functional role in coordinating cell behavior *in vivo*. It should be noted that we have not yet confirmed that the gap junctions identified in our system are functional (e.g. by performing a dye transfer assay). Nonetheless, corneal keratocytes and corneal fibroblasts within 3-D collagen matrices each exhibit a morphology, cytoskeletal organization and connectivity similar to their *in vivo* counterpart. Overall, this experimental model should provide a unique platform for quantitative assessment of the mechanical phenotypes of corneal keratocytes induced in response to various growth factors and other stimuli in a defined 3-D microenvironment.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1.

Maximum intensity projections of f-actin organization along the z-axis (A, B) or y-axis (C, D). Cells were plated in 3-D matrices and cultured for 24 hours. A&C: Corneal keratocyte. B&D: Corneal fibroblast. Keratocytes had a stellate morphology with numerous cell processes (A) and a more 3-dimensional structure (C). Fibroblasts typically had a bipolar morphology with thin pseudopodial processes (B). Stress fibers were often observed and cells were oriented nearly parallel to the bottom of the culture dish (D). (E) Quantitative analysis of cell morphology. Both the breadth/length ratio and cell height were larger for corneal keratocytes as compared to corneal fibroblasts. (* $P < 0.01$, ** $P < 0.001$)

Figure 2.

Color overlays of f-actin (green) and tubulin (red) following 24hrs of 3-D culture in serum-free media. A cortical f-actin labeling pattern was observed, and stress fibers were absent. Dendritic processes contained a core of microtubules (arrowheads) with f-actin concentrated near their tips (arrows). Filopodial extension were also observed (double arrows).

Figure 3.

Time-lapse DIC images of a corneal keratocyte (A, C) and fibroblast (B, D). Matrix deformation is indicated by red tracks (crosses mark positions prior to adding cytochalasin D). (B, D) Following culture in serum-containing media, Cytochalasin D induced fibroblast elongation and ECM relaxation. (A, B) In contrast, cytochalasin D had little impact on the matrix surrounding the corneal keratocyte, indicating much smaller cellular forces. (E, F) FEM strain maps generated from matrix displacements. Color legend is in dimensionless units $\Delta L/L$ (change in length/initial length). Note the higher strains induced by the fibroblast as compared to the keratocyte.

Figure 4.

Maximum intensity projection color overlays of f-actin (green) and collagen fibrils (red) following 24hrs of 3-D culture. (A) Keratocytes had a stellate morphology and numerous cell processes which ran a tortuous path between and along collagen fibrils without any apparent impact on their alignment. (B) Increased compaction and alignment of collagen fibrils parallel to the pseudopodial tips was observed at the ends of fibroblasts. Adjacent to the cell body, a more random collagen organization was observed in both cases. (C) Corneal fibroblasts treated with the Rho kinase inhibitor Y-27632 developed a stellate morphology with long dendritic processes, and there was a marked reduction in local collagen matrix reorganization

Figure 5.

Local collagen organization. Increased alignment of collagen fibrils at the ends of pseudopodia was observed in fibroblasts as compared to keratocytes or control matrices (no cells), as indicated by higher OI values. Adjacent to the cell body, preferential alignment of collagen was not detected under any condition evaluated. (** $P < 0.001$)

Figure 6.

Collagen fibril density. Significantly greater collagen fibril density was observed surrounding culture of fibroblasts, as compared to culture of keratocytes or control matrices (no cells). Values are % of area occupied by segmented collagen fibrils in individual optical sections. (** $P < 0.001$, * $P < 0.01$)

Figure 7.

Assessment of global matrix contraction at low cell density. Cell-induced global matrix contraction was significantly greater in fibroblasts, as compared to keratocytes and control matrices (no cells). (** $P < 0.001$, * $P < 0.01$)

Figure 8.

Cells in 3-D matrices plated at high density and cultured for 24 hours in serum-free media (A, C, E) or 10% FBS (B, D, F) and labeled with phalloidin (green) and/or connexin 43 (red). (A) Keratocytes appeared to form an interconnected network at high cell density (arrows), which was confirmed by connexin labeling (E). (C) Keratocytes were also flatter than in low density cultures, with fewer extensions along the z-axis. For fibroblasts there was an increase in the number of stress fibers as compared to low density cultures (B), and more substantial matrix compaction (D). (F) Connexin 43 was also observed between cells.

Figure 9.

Cells in 3-D matrices plated at high density and cultured for 5 days in 10% FBS and labeled with phalloidin (green) and α -SM-actin (red). Approximately 10% of cells showed positive α -SM-actin labeling localized stress fibers, suggesting myofibroblast transformation.

Figure 10.

Assessment of global matrix contraction at high cell density. Significant global matrix contraction was produced by both corneal keratocytes and fibroblasts as compared to control matrices without cells at 24 hrs ($P < 0.001$) and 5 days ($P < 0.01$). However, the amount of global matrix contraction was higher for fibroblasts at both time points ($P < 0.001$).