Growth Factor Regulation of Corneal Keratocyte Mechanical Phenotypes in 3-D Collagen Matrices

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PURPOSE. To assess how wound healing cytokines and the extracellular matrix (ECM) environment regulate the keratocyte mechanical phenotype.

METHODS. Rabbit corneal keratocytes were plated within standard bovine or rat tail type I collagen matrices (2.5 mg/mL), compressed collagen matrices (approximately 100 mg/mL), or on collagen-coated dishes and cultured for up to 7 days in serum-free media, platelet derived growth factor BB (PDGF BB), insulin-like growth factor (IGF), TGFβ1, TGFβ2, or FGF2. F-actin, α-smooth muscle actin (α-SMA) and collagen fibrils were imaged using confocal microscopy. Cell morphology, local matrix reorganization, and global matrix contraction were quantified digitally.

RESULTS. IGF and PDGF BB stimulated elongation of keratocytes and extension of dendritic processes within 3-D matrices, without inducing stress fiber formation or collagen reorganization. In contrast, treatment with TGFβ1 and TGFβ2 increased keratocyte contractility, as indicated by stress fiber formation and matrix compaction and alignment. This transformation was enhanced at higher cell densities within standard 3-D matrices, in which α-SMA was incorporated into stress fibers. In contrast, α-SMA was expressed within compressed 3-D matrices even at low cell density. FGF2 did not produce significant cytoskeletal or matrix reorganization in standard 3-D matrices; however, stress fibers were consistently expressed within compressed collagen matrices and on rigid two-dimensional substrates. Inhibiting Rho kinase blocked both TGFβ- and FGF2-induced stress fiber formation.

CONCLUSIONS. Keratocytes cultured in IGF or PDGF BB maintain a quiescent mechanical phenotype over a range of matrix environments. In contrast, the mechanical phenotypes induced by FGF and TGFβ vary in response to the structural and/or mechanical properties of the ECM. (Invest Ophthalmol Vis Sci. 2012;53:1077–1086) DOI:10.1167/iovs.11-8609

Stromal keratocytes play a central role in mediating the corneal response to lacerating injury or refractive surgery.1 During wound healing, quiescent corneal keratocytes surrounding the area of injury generally become activated, and transform into a fibroblastic repair phenotype.2,5 These activated fibroblasts proliferate, migrate into the provisional matrix, and generate the forces required for wound closure. In certain wound types, fibroblasts further differentiate into myofibroblasts, which generate even stronger forces and synthesize a fibrotic extracellular matrix (ECM) associated with scar formation.4,5 These wound healing responses can cause a permanent reduction in corneal clarity, and can also decrease the refractive effect of vision correction surgeries such as photorefractive keratectomy (PRK).6,7 Peptide growth factors present in the cornea and tear film, such as insulin-like growth factor (IGF), platelet derived growth factor (PDGF), FGF, IL-1α and TGFβ8–11 are postulated to play an important role in modulating the keratocyte phenotype during corneal wound healing. In cell culture, these growth factors differentially regulate keratocyte proliferation, cytoskeletal organization, and ECM synthesis. Keratocytes cultured under serum-free conditions maintain the quiescent, dendritic phenotype normally observed in vivo.12,13 IGF stimulates keratocyte proliferation without altering cell morphology, cytoskeletal organization, or downregulating keratocan sulfate proteoglycan (KSPG) synthesis.14,15 In contrast, exposure to serum results in fibroblast differentiation, as indicated by the assumption of a bipolar morphology, formation of intracellular stress fibers, and downregulation of keratin sulfate proteoglycan expression.15,16–18 PDGF and basic FGF (FGF2) have also been shown to activate corneal keratocytes in vitro, but have distinct effects on cell morphology, cytoskeletal organization, and proteoglycan synthesis.14,15,19,20 TGFβ induces myofibroblast differentiation, as indicated by expression of stress fibers containing α-smooth muscle actin (α-SMA),21 and production of abnormal, fibrotic ECM.15,20,22 PDGF participates in TGFβ-induced myofibroblast differentiation through an autocrine feedback loop,23 whereas FGF2 has been shown to reduce expression of α-SMA.24

Most previous studies investigating the effect of these growth factors on corneal keratocyte differentiation have been performed using rigid, two-dimensional (2-D) substrates. However, keratocytes reside within a complex 3-D extracellular matrix in vivo, and significant differences in cell morphology, adhesion organization, and mechanical behavior have been identified in 2-D versus 3-D culture models.25–27 Furthermore, unlike rigid 2-D substrates, 3-D models also allow assessment of cellular force generation and cell-induced matrix reorganization; biomechanical activities that are critically involved in the migratory, contractile, and remodeling phases of wound healing. Recent studies also suggest a potential linkage between increased keratocyte contractility and altered ECM synthesis, further highlighting the potential importance of cell mechanics in the wound repair process.20,28 We have recently established 3-D culture models which support keratocyte differentiation in serum-free media, and allow changes in cell morphology, mechanical activity, and matrix reorganization to be measured using quantitative imaging techniques.29,30 In this study, we use these models to assess the effects of PDGF BB, FGF2, IGF, TGFβ1, and TGFβ2 on the keratocyte mechanical phenotype.
in 3-D collagen matrices, and compare these responses with those observed on a rigid 2-D substrate.

**METHODS**

**Cell Culture**

Corneal keratocytes were isolated from rabbit eyes obtained from Pel Freez (Rogers, AR) as previously described. Cells were cultured in flasks with serum-free medium (basal medium) consisting of Dulbecco’s modified Eagle’s minimum essential medium with pyruvate (DMEM; Invitrogen, Carlsbad, CA), supplemented with 1% RPMI vitamin mix (Sigma-Aldrich, St. Louis, MO), 100 μM nonessential amino acids (Invitrogen), 100 μg/mL ascorbic acid, and 1% penicillin/streptomycin amphotericin B (Fungizone; BioWhittaker, Inc., Walkersville, MD) to maintain the keratocyte phenotype.14

**Preparation of Standard (Uncompressed) Collagen Matrices**

Hydrated collagen matrices were prepared by mixing neutralized bovine dermal collagen (Purecol; Inamed, Fremont, CA) or type I rat tail collagen (BD Biosciences, San Jose, CA) with 10 × DMEM to achieve a final collagen concentration of 2.5 mg/mL.29 A 50 μL of suspension of cells was then mixed with the above collagen solution. After adjusting the pH to 7.2 by addition of NaOH, 30 μL aliquots of the cell/collagen mixture were spread over a central 12 mm diameter circular region on culture dishes (Delta T; Biopetchs, Inc., Butler, PA). The dishes were then placed in a humidified incubator for 60 minutes for dermal collagen matrices or 30 minutes for type I rat tail collagen matrices for polymerization. The matrices were overlaid with 1.5 mL of serum-free medium (basal media). After 24 hours of incubation to allow cell spreading, the media was replaced with basal media or basal media supplemented with 10 ng/mL FGF2 (Sigma-Aldrich), 10 ng/mL IGF-I (Sigma-Aldrich), 50 ng/mL PDGF BB (Millipore Corp, Temecula, CA), 10 ng/mL TGFβ1 or TGFβ2 (Sigma-Aldrich), and cultured for an additional 4 days. Growth factor concentrations were determined from preliminary dose-response experiments and represent the lowest concentration to give a maximal effect on changes in cell morphology and F-actin organization. Matrices with both low cell density (2 × 10^5 cells per matrix) and high cell density (5 × 10^5 cells per matrix) were used.

**Confocal Imaging**

After 4 days of culture in test media, cells were fixed using 3% paraformaldehyde in phosphate buffer for 15 minutes and permeabilized with 0.5% Triton X-100 in phosphate buffer for 3 minutes. To label F-actin, Alexa Fluor 488 phalloidin was used (1:20, Invitrogen). In other experiments immunolabeling with α-SMA was performed. After incubation in 1% BSA for 60 minutes to block nonspecific binding, cells were incubated for 1 hour in mouse monoclonal antibodies to α-SMA (1:100, Sigma-Aldrich) in 1% BSA. Cells were then washed in buffer and incubated for 1 hour in affinity-purified Rhodamine conjugated goat anti-mouse IgG (1:100, Sigma-Aldrich) in 1% BSA. The dishes were then placed in a humidified incubator for 60 minutes to allow cell attachment. The media was then removed and 2 mL basal media was added to each dish. After 24 hours of incubation, the media was changed to basal media or basal media supplemented with growth factors, and cultured for an additional 4 to 7 days before fixation and staining as described above.

**Global Matrix Contraction**

Differential interference contrast (DIC) imaging was used to measure global matrix contraction of standard collagen matrices. Because the bottom of the matrices remain attached to the dish, cell-induced contraction results in a decrease in matrix height.32 Height was measured by focusing on the top and bottom of each matrix at six different locations. Measurements were performed in triplicate for each condition, and repeated three times. The percentage decrease in matrix height over time was then calculated.

**Assessment of Local Collagen Fibril Orientation**

To assess the amount of local cell-induced ECM reorganization within standard collagen matrices, the density of collagen surrounding cells was assessed from reflected light confocal images as previously described.29,31 Briefly, three to four subregions surrounding each cell were outlined, and the percent of each subregion occupied by collagen fibrils was used as an indicator of collagen fibril density. Using software (MetaMorph; Molecular Devices), 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. Measurements were performed on a minimum of 10 cells for each condition, taken from three separate experiments.

**2-D Culture Experiments**

Cell suspensions containing 300 or 10,000 rabbit corneal keratocytes cells in basal media were added to collagen-coated dishes with 10 mm diameter microwells (MatTek Corporation, Ashland, MA), and left for 30 minutes to allow for cell attachment. The media was then removed from the well and 2 mL basal media was added to each dish. After 24 hours of incubation, the media was changed to basal media or basal media supplemented with growth factors, and cultured for an additional 4 to 7 days before fixation and staining as described above.

**Compressed Collagen Matrices**

Compressed collagen matrices were prepared as described previously by Brown and coworkers.33,34 Briefly, 10 mg/mL of type I rat tail collagen (BD Biosciences) was diluted to a final concentration of 2 mg/mL. After drop-wise neutralization with 1 M sodium hydroxide, a suspension of 2 × 10^5 or 2 × 10^6 keratocytes in 0.6 mL basal media was added to the collagen mixture. The solution containing cells and the collagen was poured into a 3 × 2 × 1 cm stainless steel mold and allowed to set for 30 minutes at 37°C. To compact the matrices, a layer of nylon mesh (approximately 50 μm mesh size) was placed on a double layer of filter paper. The matrices were placed on the nylon mesh, covered with a pane of glass, and loaded with a 130 g stainless steel block for 5 minutes at room temperature. This process squeezes media out of the matrix and results in the formation of a flat, cell/collagen sheet with high mechanical stiffness. After compression, 6 mm diameter buttons were punched out of the matrix using a trephine. After 24 hours of incubation to allow cell spreading, the media was replaced with basal media or basal media supplemented with 10 ng/mL FGF2, 10 ng/mL IGF, 50 ng/mL PDGF BB, or 10 ng/mL TGFβ1, with or without the addition of the Rho kinase inhibitor Y-27632 (10 μM), and buttons were cultured for an additional 4 days. Confocal imaging was then performed as described above. Experiments were performed using duplicate samples, and repeated three times.
Statistics

Statistical analyses were performed using commercial software (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), or ANOVA on ranks (for nonparametric data) was used to compare group means, and post hoc multiple comparisons were performed using the Holm-Sidak method (for parametric data) or Dunn’s method (for nonparametric data). Differences were considered significant if $P < 0.05$.

RESULTS

Standard (Uncompressed) Collagen Matrices

Cell Morphology and Cytoskeletal Organization. After 4 days of culture in basal media, keratocytes in bovine collagen matrices maintained a broad, convoluted cell body with numerous thin dendritic processes. They had a cortical, membrane associated F-actin organization, with more concentrated labeling near the ends of cell processes. Stress fibers were rarely observed (Fig. 1A). This morphology and cytoskeletal organization is consistent with that of quiescent corneal keratocytes in vivo.13

Keratocytes exposed to FGF2 appeared to maintain a normal dendritic/stellate morphology (Fig. 1B). The organization of F-actin likewise maintained a cortical localization predominantly distributed along the cell membrane, and stress fibers were not observed. There was, however, an apparent increase in ruffling at the tips of cell processes. Keratocytes treated with PDGF BB and IGF were much more elongated and developed thin dendritic processes (Figs. 1C and 1D). In contrast, keratocytes treated with TGFβ1 and TGFβ2 lost dendritic processes and developed a more spread morphology. Within the cell body, F-actin filament bundles (stress fibers) were observed in most cells (Figs. 1E and 1F). However, these stress fibers were not as prominent as those typically observed on rigid, 2-D substrates.35 Quantitative analysis of cell morphology of isolated keratocytes from low cell density matrices confirmed that IGF, PDGF BB, and FGF2 stimulated an increase in cell length and/or height (Fig. 2). In contrast, TGFβ1 and TGFβ2 induced a reduction in cell height, indicating fewer cell extensions along the z-axis.

Previous studies have demonstrated that nonpepsinized rat tail collagen forms shorter fibrils, and has smaller pores and a higher fiber density compared with pepsin-extracted bovine collagen.36 Despite these differences in matrix structure, the

FIGURE 1. Maximum intensity projections of F-actin organization. Cells were plated at low cell density in standard (uncompressed) 3-D bovine dermal collagen matrices and cultured with different growth factors for 4 days. (A) Corneal keratocytes in basal media had a stellate morphology with dendritic cell processes. F-actin labeling was generally limited to the cell cortex. (B) Keratocytes cultured in FGF2 had a similar morphology, but exhibited ruffling at the tips of cell processes. (C) Cells treated with IGF were elongated and formed thin dendritic extensions (note change in scale bar). (D) Cells treated with PDGF BB also appeared more elongated with dendritic cell processes. (E, F) Cells treated with TGFβ1 or TGFβ2 generally developed a more spread morphology with most cells expressing F-actin filament bundles (stress fibers). The x-z projections show fewer extensions in the z-axis under TGFβ1 and TGFβ2 culture conditions.

FIGURE 2. Quantitative analysis of cell morphology in standard bovine collagen matrices. IGF, PDGF BB, and FGF2 stimulated an increase in cell length and/or height. In contrast, TGFβ1 and TGFβ2 induced a reduction in cell height, indicating fewer cell extensions along the z-axis.
changes in cell morphology and cytoskeletal organization under the different conditions studied was similar to that observed in bovine collagen (not shown).

**Local Cell-Induced Matrix Reorganization.** 3-D confocal images of isolated keratocytes from low cell density matrices were used to analyze the local collagen matrix reorganization after 4 days of incubation with growth factors. Fluorescent imaging was used to visualize f-actin and reflected light imaging was used to visualize collagen surrounding cells. In general, minimal compaction and/or realignment of collagen fibrils was observed surrounding cells treated with FGF2, IGF, PDGF BB, or control conditions (Figs. 3A–D, 3G). In contrast, collagen surrounding cells in TGFβ1 and TGFβ2 appeared to be more compacted, in both bovine (Figs. 3E, 3F) and rat tail matrices (Figs. 3H, 3I). Quantitative analysis demonstrated that collagen density was significantly higher for TGFβ1 and TGFβ2 compared with keratocytes treated with FGF2, IGF, and PDGF BB also did not appear to produce significant collagen reorganization. Increased compaction of collagen fibrils parallel to the pseudopodial tips and adjacent to the cell body was observed after culture in both TGFβ1 and TGFβ2. This alignment was generally more apparent in rat tail collagen (G–I).

**Effect of Cell Density on Keratocyte Phenotype.** At higher cell density, keratocytes in basal media were flatter than in low density cultures, and the dendritic processes of adjacent cells appeared to interconnect. This interconnected network was confirmed previously using Connexin 43 labeling. Cells treated with FGF2, IGF, and PDGF BB also developed interconnected dendritic processes, and stress fibers were not observed (Fig. 5). Consistent with previous results, a small increase in global matrix contraction was produced by keratocytes in basal media compared with control matrices without cells, in both bovine and rat tail matrices (Fig. 6); indicating that keratocytes are not completely devoid of mechanical activity. Low levels of global matrix contraction were also observed after culture in FGF2, IGF, and PDGF BB (Fig. 6). In contrast, cells treated with TGFβ1 and TGFβ2 had a striking increase in the number of stress fibers compared with low density cultures (Fig. 5), and the collagen appeared much more compacted by confocal reflection imaging (not shown). These responses were observed in both bovine and rat tail collagen matrices. Consistent with these results, significant global matrix contraction was produced by both TGFβ1- and TGFβ2-treated corneal keratocytes compared with other conditions and matrices without cells, in both bovine and rat tail matrices (Fig. 6).

To determine whether TGFβ induced myofibroblast transformation, α-SMA staining was performed. Approximately 20%
of cells showed positive labeling for α-SMA localized to the stress fibers at high cell density (Fig. 7); in contrast, α-SMA labeling was negative for TGFβ1- and TGFβ2-treated cells plated at low cell density (not shown).

2-D Culture

To assess potential differences in growth factor responses between 2-D and 3-D environments, studies were also performed using collagen-coated culture dishes. In basal media, keratocytes developed a dendritic morphology with membrane associated f-actin labeling, consistent with previous results (Fig. 8A). After culture in media containing IGF (Fig. 8B) or PDGF BB (Figs. 8C and 8E), cells maintained this quiescent phenotype at both high and low cell density, similar to the results in 3-D matrices. At low cell density, PDGF BB keratocyte elongation via extension of thin dendritic processes could be appreciated (Fig. 8E). In contrast, FGF2 induced a switch from a dendritic morphology to a spread morphology, and prominent stress fiber bundles were observed at both low (Fig. 8F) and high (Fig. 8D) cell density. These responses were observed at both 4 and 7 days of culture. TGFβ induced myofibroblast transformation, as indicated by loss of dendritic processes, and development of stress fibers containing α-SMA (Figs. 8E, 8F). Consistent with previous observations, α-SMA was incorporated into stress fibers in approximately 60% of cells after 4 days of culture in TGFβ.

Compressed Collagen Matrices

The mechanical stiffness of rigid 2-D substrates are many orders of magnitude higher than standard collagen matrices. Thus the fibroblastic transformation induced by FGF2 and increased α-SMA expression induced by TGFβ in 2-D culture may be the result of increased stiffness, and not ECM dimensionality (2-D versus 3-D). To investigate the role of ECM stiffness on keratocyte responses, we plated cells within compressed collagen matrices, which provide a much stiffer 3-D culture environment than standard collagen matrices. Keratocytes in compressed collagen matrices cultured in serum-free media developed a dendritic morphology, as previously
reported. In contrast, FGF2 induced a switch to a spread morphology, and prominent stress fiber bundles were consistently observed at both 1 day (Fig. 9A) and 4 days after treatment (Fig. 9B). TGF\beta also induced loss of dendritic processes and stress fiber formation as early as 1 day after plating (Fig. 9D). By 4 days, TGF\beta induced myofibroblast transformation, as indicated by stress fibers containing \alpha-SMA (Fig. 9E). While stress fibers were observed in all cells irrespective of cell density, the percentage of cells with \alpha-SMA incorporated into stress fibers was much greater at higher cell density (60% versus 20%).

The Rho-family of GTPases, such as Rho and Rac, play a central role in the regulation of cell morphology, cytoskeletal organization, and global contraction of 3-D collagen matrices. Rho is known to promote increased phosphorylation of myosin light chain via Rho-kinase (ROCK) inhibition of myosin light chain phosphatase (MLCPase), resulting in increased actin-myosin II-based cell contractility. We previously demonstrated that Rho kinase plays a central role in regulating corneal fibroblast contractility and matrix reorganization within standard 3-D matrices. In 2-D culture, Rho kinase has also been shown to mediate fibroblastic and myofibroblastic transformation of keratocytes in response to FGF2 and TGF\beta treatment, respectively. To determine whether Rho kinase plays a role in transformation of keratocytes in compressed 3-D matrices, we used the established Rho kinase inhibitor Y-27632. Treatment with Y-27632 blocked the induction of stress fibers normally induced by both FGF2 (Fig. 9C) and TGF\beta (Fig. 9F).

**DISCUSSION**

While much is known regarding the biochemical and morphologic characteristics of corneal keratocytes and fibroblasts, less
is known about how the mechanical behavior of these cells is regulated. In this study, we assessed the effects of several growth factors on the keratocyte mechanical phenotype using a standard 3-D fibrillar collagen matrix model. In this model, matrix contraction occurs by compaction of collagen fibrils through the application of cellular forces. Assessment of overall matrix contraction is a standard assay for assessing changes in cell contractility in response to different culture conditions. However, such global measurements can be limited, because they do not provide information on the changes in 3-D cell morphology, cytoskeletal organization, or local cell-induced matrix reorganization which reflect the mechanical state of cells. In this study, we performed a comprehensive assessment of the keratocyte mechanical response to IGF, PDGF BB, FGF2, TGFβ1, and TGFβ2, using high magnification 3-D confocal imaging. We then compared these responses to those observed on rigid 2-D substrates or within compressed collagen matrices. These growth factors were selected because they are present in the cornea and/or tear film, and have already been shown to regulate keratocyte proliferation and ECM synthesis in vitro.

Consistent with previous studies, corneal keratocytes maintained in basal, serum-free media had a dendritic morphology in both standard and compressed 3-D matrices, and do not express stress fibers or produce large amounts of matrix reorganization. At higher cell densities, these dendritic processes interconnect, producing a lattice-like network similar to that observed in vivo. Dermal fibroblasts form similar dendritic cell processes when contractility is blocked by inhibiting myosin II, and serum-cultured corneal fibroblasts develop dendritic processes in response to Rho kinase inhibition. Thus the dendritic cell morphology appears to be a hallmark of cells in a low tension environment. IGF induced elongation of keratocyte dendritic processes without producing significant collagen matrix reorganization in 3-D culture. A similar morphology and cytoskeletal organization was observed on a collagen-coated 2-D substrate, consistent with previous observations. Thus overall, keratocytes cultured in IGF maintained a quiescent mechanical phenotype. IGF has been shown by others to increase keratocyte proliferation and to stimulate synthesis of ECM components resembling normal corneal stroma, and also to stimulate network formation. Thus it has been suggested that IGF may be involved in maintenance of normal corneal structure and could contribute to a regenerative wound healing phenotype. Our data demonstrating keratocytes cultured in IGF maintain a quiescent mechanical phenotype in 3-D culture are consistent with this hypothesis.

PDGF BB also induced keratocyte elongation and formation of dendritic processes in both 2-D and 3-D culture, without producing stress fibers or significant matrix reorganization. PDGF BB has been shown previously to stimulate Rac-induced spreading of dermal and corneal fibroblasts in 3-D collagen matrices, along with significant tractional force generation by extending pseudopodial processes. However, fibroblasts...
in those studies were maintained in serum, and thus had a significant basal level of Rho kinase activity. When Rho kinase is inhibited, corneal fibroblasts revert to a dendritic morphology, and only small collagen displacements are observed during PDGF BB-induced cell spreading, consistent with our results in the present study using quiescent corneal keratocytes.56 Like IGF, PDGF stimulates keratocyte proliferation and has been shown to upregulate synthesis of normal stromal ECM.14,23 In addition, PDGF BB has been shown to be a potent stimulator of both corneal keratocyte and dermal fibroblast migration.8,50,52–54 Thus it is interesting to speculate that PDGF BB may contribute to stromal repopulation after injury or surgery through upregulation of both proliferation and migration, without producing fibrotic tissue or generating large forces which can alter corneal shape and transparency.

Interestingly, PDGF AB has been shown to transform corneal keratocytes to a fibroblastic phenotype in 2-D culture, as indicated by the development of stress fibers and focal adhesions.14,23 However, this transformation was not observed in response to PDGF BB under any of the conditions used in the present study (including 2-D substrates). PDGF AB has also been shown to stimulate contraction of floating collagen matrices in 3-D culture. However, it should be noted that contraction of floating matrices occurs through a Rho kinase-independent mechanism, and does not require generation of significant cellular forces. In contrast, contraction of attached collagen matrices requires larger forces and has been shown to be Rho kinase-dependent.32

Previous studies indicate that FGF2 induces fibroblastic transformation of keratocytes on 2-D substrates, as indicated by changes in cell morphology and development of stress fibers and focal adhesions.14,23 We observed a similar response in this study when keratocytes were cultured on collagen-coated 2-D substrates or within compressed collagen matrices. However, in standard 3-D matrices, FGF2 appeared to stimulate ruffling of keratocyte processes without inducing major changes in cell morphology or collagen matrix organization. Furthermore, FGF2 did not induce formation of stress fibers in 3-D matrices, even at high cell density. Thus unlike PDGF BB, the response of keratocytes to FGF2 appears to be sensitive to ECM mechanical stiffness and/or ligand density. It is well known that increasing substrate stiffness can facilitate formation of actin stress fibers and focal adhesions in contractile cells.55–57 Both glass and compressed collagen matrices are several orders of magnitude stiffer than a hydrated collagen matrix, and this may underlie the difference in mechanical phenotype induced by FGF2. In the cornea, large shifts in the global distribution of ECM tension can be induced by lacerating injury, penetrating keratoplasty, or refractive surgery.5 In addition, the provisional wound healing matrix is less dense, more disorganized, and more compliant than normal corneal tissue. The response of corneal keratocytes to FGF2 during wound healing could be regulated, in part, by such changes in ECM composition, stress, and stiffness.

Consistent with previous studies, treatment with TGFβ1 and TGFβ2 increased cell contractility within standard 3-D matrices, as indicated by the formation of stress fibers and stimulation of cell-induced ECM reorganization.5,14 Interestingly, these changes were significantly enhanced at higher cell densities. Approximately 20% of cells showed positive labeling for α-SMA localized to the stress fibers at high cell density; in contrast, α-SMA labeling was negative for TGFβ1- and TGFβ2-treated cells plated at low cell density. Both connective tissue growth factor (CTGF) and PDGF have been shown to participate in TGFβ-induced myofibroblast differentiation through an autocrine feedback loop, which would be amplified at higher cell density.23–58 In addition, mechanical cross-talk between cells increases the tension within the matrix at higher cell density, and ECM stiffness is known to upregulate myofibroblast transformation.59 A recent study demonstrated that increased matrix stiffness enhances TGFβ-induced myofibroblast transformation of human tenon fibroblasts in 2-D culture.60 We observed a similar increase in stress fiber formation and myofibroblast transformation of corneal keratocytes on both rigid 2-D substrates and within compressed 3-D collagen matrix. While stress fibers were observed in all cells within compressed ECM irrespective of cell density, the percentage of cells with α-SMA incorporated into stress fibers was greater at higher cell density (approximately 60% versus approximately 20%), suggesting that both mechanical stiffness and autocrine signaling promote myofibroblast transformation. Interestingly, myofibroblasts tend to develop toward the end of the corneal wound repair process when cell density is high and the wound environment is stiffer, suggesting similar processes may be involved during in vivo healing.

FIGURE 9. Corneal keratocytes plated within compressed collagen matrices and cultured in FGF2 (A–C) or TGFβ1 (D–F) for 1 (A, C, D, F) or 4 (B, E) days. Keratocytes treated with FGF2 or TGFβ consistently developed prominent stress fibers. In TGFβ, many of the cells also expressed α-SMA (E, green, F-actin; red, α-SMA). Stress fiber formation was blocked when Rho kinase was inhibited using Y-27632 (C, F).
We have previously shown that Rho kinase plays a central role in mediating cellular force generation and matrix reorganization by serum cultured corneal fibroblasts in 3-D culture. In 2-D culture, Rho kinase has also been shown to mediate fibroblastic transformation of keratocytes in response to FGF2 treatment, and myofibroblast transformation in response to TGFβ. In the present study, we demonstrated that Rho kinase is also required for transformation of keratocytes within compressed 3-D matrices. Interestingly, blocking Rho/Rho kinase has been shown to inhibit the decrease in KSPG synthesis normally associated with myofibroblast transformation, suggesting a linkage between increased cell contractility and fibrotic ECM synthesis.

3-D culture models typically use either bovine dermal collagen (which is pepsinized), or rat tail tendon collagen (which is not pepsinized). Pepsin treatment reductively cleaves cross-link mediating telopeptides from collagen monomers, which alters the structural and mechanical properties of reassembled collagen matrices. Nonpepsinized rat tail collagen forms shorter fibrils, and has smaller pores and a higher fiber density compared with pepsin-extracted bovine collagen. Recent studies have shown that both the mechanics and protease dependency of migration by certain tumor cell lines is impacted by the type of collagen used.

In the present study however, the morphologic and mechanical responses of corneal keratocytes to growth factor treatments were remarkably similar for these two matrix types. Taken together, the data demonstrate that the keratocyte mechanical phenotypes induced by growth factors can be differentially regulated by ECM structure and/or mechanical properties. Most notably, whereas FGF2 induces a contractile fibroblastic phenotype on rigid 2-D substrate or compressed collagen ECM, a quiescent mechanical phenotype is observed in standard 3-D matrices. Furthermore, while TGFβ collagen ECM, a quiescent mechanical phenotype is observed fibroblastic phenotype on rigid 2-D substrate or compressed compressed ECM, a quiescent mechanical phenotype is observed. Keratocytes cultured in IGF or PDGF BB consistently maintain a quiescent mechanical phenotype over a range of matrix environments, this transformation appears to be enhanced by both increased substrate stiffness and autocrine signaling. Keratocytes cultured in IGF or PDGF BB consistently maintain a quiescent mechanical phenotype over a range of matrix environments, and may thus have the potential to modulate migration, proliferation, and/or ECM synthesis during wound healing, without generating large contractile forces which can disrupt normal corneal structure and transparency.

References


