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

Neema Lakshman and W. Matthew Petroll

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), TGF β 1, TGF β 2, 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 TGF β 1 and TGF β 2 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 TGF β - 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 TGF β 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.^{2,3} These activated fibroblasts proliferate, migrate into the provisional ma-

trix, 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-1 α and TGF β ,⁸⁻¹¹ 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 keratan 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.^{13,16-18} 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} TGF β 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 TGF β -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, TGF β 1, and TGF β 2 on the keratocyte mechanical phenotype

From the Department of Ophthalmology, University of Texas Southwestern Medical Center, Dallas, Texas.

Supported in part by Grants NIH R01 EY 013322, NIH P30 EY020799, and an unrestricted grant and Senior Scientific Investigator Award (WMP) from Research to Prevent Blindness, Inc., New York, NY.

Submitted for publication September 16, 2011; revised December 7, 2011, and January 3, 2012; accepted January 3, 2012.

Disclosure: N. Lakshman, None; W.M. Petroll, None

Corresponding author: W. Matthew Petroll, Department of Ophthalmology, Southwestern Medical Center, 5323 Harry Hines Boulevard, Dallas, TX 75390-9057; matthew.petroll@utsouthwestern.edu.

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 μ M nonessential amino acids (Invitrogen), 100 μ g/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 $\times$ DMEM to achieve a final collagen concentration of 2.5 mg/mL.²⁹ A 50 μ L of suspension of cells was then mixed with the above collagen solution. After adjusting the pH to 7.2 by addition of NaOH, 30 μ L aliquots of the cell/collagen mixture were spread over a central 12 mm diameter circular region on culture dishes (Delta T; Biopetech, 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 media (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 TGF β 1 or TGF β 2 (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 actin organization. Matrices with both low cell density (2×10^3 cells per matrix) and high cell density (5×10^4 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 (1:20, MP Biomedicals, Solon, OH).

Constructs were imaged using laser scanning confocal microscopy (Leica SP2, Heidelberg, Germany) as previously described.³¹ 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). Changes in cell morphology were measured using computer software (MetaMorph; Molecular Devices, Sunnyvale, CA). Sequential scanning was used to image double-labeled samples to prevent cross-talk between fluorophores.

Cell Morphology

Changes in cell morphology within standard collagen matrices were measured using image analysis software (MetaMorph; Molecular Devices) as previously described.³¹ Projected cell length 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 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. Measurements were performed on a minimum of 10 cells for each condition, taken from three separate experiments.

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 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 2×10^4 or 2×10^5 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 $\times$ 2 $\times$ 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 μ m 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 TGF β 1, with or without the addition of the Rho kinase inhibitor Y-27632 (10 μ M), 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.

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 TGF β 1 or TGF β 2 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 TGF β 1 and TGF β 2 culture conditions.

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 end 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 TGF β 1 and TGF β 2 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, TGF β 1 and TGF β 2 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 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, TGF β 1 and TGF β 2 induced a reduction in cell height, indicating fewer cell extensions along the z-axis.

FIGURE 3. Maximum intensity projections of f-actin (green) and collagen fibrils (red) after 4 days of culture in standard 3-D matrices. (A–F) Bovine collagen; (G–I) rat tail collagen. (A, G) After culture in basal media, collagen fibrils were randomly oriented, and significant alignment or compaction of fibrils at the end of cells was not generally observed. (B–D) Keratocytes treated with FGF2, IGF, and PDGF BB also did not appear to produce significant matrix reorganization. (E, F) Increased compaction of collagen fibrils parallel to the pseudopodial tips and adjacent to the cell body was observed after culture in both TGF β 1 and TGF β 2. This alignment was generally more apparent in rat tail collagen (G–I).

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.³¹ 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 TGF β 1 and TGF β 2 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 TGF β 1 and TGF β 2 compared with keratocytes treated with FGF2, IGF, PDGF BB, or basal media in both bovine and rat tail collagen matrices (Fig. 4).

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.²⁹ 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 collagen matrices (Fig. 6)²⁹; indicating that keratocytes are not completely devoid of mechanical activity.²⁹ Low levels of global matrix contraction were also observed after culture in FGF2, IGF, and PDGF BB (Fig. 6). In contrast,

cells treated with TGF β 1 and TGF β 2 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 TGF β 1- and TGF β 2-treated corneal keratocytes compared with other conditions and matrices without cells, in both bovine and rat tail matrices (Fig. 6).

To determine whether TGF β induced myofibroblast transformation, α -SMA staining was performed. Approximately 20%

FIGURE 4. Local collagen fibril density within standard 3-D matrices. Significantly greater collagen fibril density was observed surrounding cells in TGF β 1 and TGF β 2, compared with all other conditions. Values are percent of area occupied by segmented collagen fibrils in individual optical sections.

FIGURE 5. F-actin organization for keratocytes plated a high cell density in standard bovine (A–C, G–I) and rat tail (D–F, J–L) collagen matrices. Keratocytes in basal media maintained stellate morphology, and the dendritic processes of adjacent cells were interconnected. Cells treated with FGF2, IGF, and PDGF BB also appeared to develop interconnected dendritic processes, and stress fibers were not observed. In contrast, cells treated with TGF β 1 and TGF β 2 had a spread or bipolar morphology, and a striking increase in the number of stress fibers. Results were similar in both bovine and rat tail collagen.

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 TGF β 1- and TGF β 2-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 factin labeling, consistent with previous results (Fig. 8A).¹³ 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. TGF β 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 TGF β .^{23,35}

Compressed Collagen Matrices

The mechanical stiffness of rigid 2-D substrates are many orders of magnitude higher than standard collagen matrices.²⁷ Thus the fibroblastic transformation induced by FGF2 and increased α -SMA expression induced by TGF β 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.³³ Keratocytes in compressed collagen matrices cultured in serum-free media developed a dendritic morphology, as previously

FIGURE 6. Assessment of global contraction of bovine dermal (A) and rat tail (B) standard collagen matrices at high cell density. In both matrix types, cell-induced matrix contraction was significantly larger for both TGFβ1 and TGFβ2, compared with all other conditions evaluated. dys, days; hrs, hours.

reported.³⁰ 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). TGFβ also induced loss of dendritic processes and stress fiber formation as early as 1 day after plating (Fig. 9D). By 4 days, TGFβ induced myofibroblast transformation, as indicated by stress fibers containing α-SMA (Fig. 9E). While stress fibers were observed in all cells irrespective of cell density, the percentage of cells with α-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.^{37,38} We previously demonstrated that Rho kinase plays a central role in regulating corneal fibroblast contractility and matrix reorganization within standard 3-D matrices.^{31,39–42} In 2-D culture, Rho kinase has also been shown to mediate fibroblastic and myofibroblastic transformation of keratocytes in response to FGF2 and TGFβ treatment, respectively.²⁰ 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 TGFβ (Fig. 9F).

DISCUSSION

While much is known regarding the biochemical and morphologic characteristics of corneal keratocytes and fibroblasts, less

FIGURE 7. Cells in standard 3-D matrices plated at high density and treated with TGF β 1 and TGF β 2 for 4 days, and labeled with phalloidin (green) and α -SMA (red). Approximately 20% of cells showed positive α -SMA labeling localized stress fibers, suggesting myofibroblast transformation.

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.^{43–46} 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.³² 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, TGF β 1, and TGF β 2, 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*.²⁹ Dermal fibroblasts form similar dendritic cell processes when contractility is blocked by inhibiting myosin II,⁴⁷ and serum-cultured corneal fibroblasts develop dendritic processes in response to Rho kinase inhibition.³¹ Thus the dendritic cell morphology appears to be a hallmark of cells in a low tension environment.⁴⁸ 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.¹⁴ 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.^{14,15,49} 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.^{14,15} 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.^{50,51} However, fibroblasts

FIGURE 8. Corneal keratocytes plated on 2-D substrates and cultured in basal media (A), IGF (B), PDGF BB (C, E), FGF2 (D, F), or TGF β 1 (G, H). Keratocytes treated with IGF or PDGF BB were elongated and dendritic. Keratocytes treated with FGF2 or TGF β developed a more spread morphology and developed stress fibers. α -SMA was incorporated into stress fibers in approximately 60% of cells after 4 days of culture in TGF. Green, f-actin; red, α -SMA.

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).

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.⁵⁰ Like IGF, PDGF stimulates keratocyte proliferation and has been shown to upregulate synthesis of normal stromal ECM.^{14,15} In addition, PDGF BB has been shown to be a potent stimulator of both corneal keratocyte and dermal fibroblast migration.^{8,30,52–54} 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.⁷ 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.^{3,14} 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.

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.³¹ 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 TGF β .²⁰ 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.²⁰

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.³⁶ Nonpepsinized rat tail collagen forms shorter fibrils, and has smaller pores and a higher fiber density compared with pepsin-extracted bovine collagen.³⁶ 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.^{36,61,62} 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 TGF β stimulates keratocyte contractility and myofibroblast transformation over a range of ECM 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

- Netto MV, Mohan RR, Ambrosio R, Hutcheon AEK, Zieske JD, Wilson SE. Wound healing in the cornea: a review of refractive surgery complications and new prospects for therapy. *Cornea*. 2005;24:509–522.
- Jester JV, Petroll WM, Cavanagh HD. Corneal stromal wound healing in refractive surgery: the role of the myofibroblast. *Prog Retinal Eye Res*. 1999;18:311–356.
- Stramer BM, Zieske JD, Jung J-C, Austin JS, Fini ME. Molecular mechanisms controlling the fibrotic repair phenotype in cornea: implications for surgical outcomes. *Invest Ophthalmol Vis Sci*. 2003;44:4237–4246.
- Jester JV, Huang J, Barry-Lane PA, Kao WW, Petroll WM, Cavanagh HD. Transforming growth factor(beta)-mediated corneal myofibroblast differentiation requires actin and fibronectin assembly. *Invest Ophthalmol Vis Sci*. 1999;40:1959–1967.
- Blalock TD, Duncan MR, Varela JC, et al. Connective tissue growth factor expression and action in human corneal fibroblast cultures and rat corneas after photorefractive keratectomy. *Invest Ophthalmol Vis Sci*. 2003;44:1879–1887.
- Moller-Pedersen T, Cavanagh HD, Petroll WM, Jester JV. Stromal wound healing explains refractive instability and haze development after photorefractive keratectomy: a 1-year confocal microscopic study. *Ophthalmology*. 2000;107:1235–1245.
- Dupps WJ, Wilson SE. Biomechanics and wound healing in the cornea. *Exp Eye Res*. 2006;83:709–720.
- Kim W-J, Mohan RR, Mohan RR, Wilson SE. Effect of PDGF, IL-1 α , and BMP2/4 on corneal fibroblast chemotaxis: expression of the platelet-derived growth factor system in the cornea. *Invest Ophthalmol Vis Sci*. 1999;40:1364–1372.
- Musselmann K, Kane BP, Alexandrou B, Hassell JR. IGF-II is present in bovine corneal stroma and activates keratocytes to proliferate in vitro. *Exp Eye Res*. 2008;86:506–511.
- Arnold DR, Moshayedi P, Schoen TJ, Jones BE, Chader GJ, Waldbillig RJ. Distribution of IGF-I and -II, IGF binding proteins (IGFBPs) and IGFBP mRNA in ocular fluids and tissues: potential sites of synthesis of IGFBPs in aqueous and vitreous. *Exp Eye Res*. 1993;56:555–565.
- Tuominen IS, Tervo TM, Teppo AM, Valle TU, Gronhagen-Riska C, Vesaluoma MH. Human tear fluid PDGF-BB, TNF-alpha and TGF-beta1 vs corneal haze and regeneration of corneal epithelium and subbasal nerve plexus after PRK. *Exp Eye Res*. 2001;72:631–641.
- Beales MP, Funderburgh JL, Jester JV, Hassell JR. Proteoglycan synthesis by bovine keratocytes and corneal fibroblasts: maintenance of the keratocyte phenotype in culture. *Invest Ophthalmol Vis Sci*. 1999;40:1658–1663.
- Jester JV, Barry PA, Lind GJ, Petroll WM, Garana R, Cavanagh HD. Corneal keratocytes: in situ and in vitro organization of cytoskeletal contractile proteins. *Invest Ophthalmol Vis Sci*. 1994;35:730–743.
- Jester JV, Chang J-H. Modulation of cultured corneal keratocyte phenotype by growth factors/cytokines control in vitro contractility and extracellular matrix contraction. *Exp Eye Res*. 2003;77:581–592.
- Etheredge L, Kane BP, Hassell JR. The effect of growth factor signaling on keratocytes in vitro and its relationship to the phases of stromal wound repair. *Invest Ophthalmol Vis Sci*. 2009;50:3128–3136.
- Dahl IMS. Biosynthesis of proteoglycans and hylauronate in rabbit corneal fibroblast cultures: variation with age of the cell line and effect of fetal calf serum. *Exp Eye Res*. 1981;32:419–433.
- Hassell JR, Schrecengost PK, Rada JA, Sundaraj N, Soti G, Thot RA. Biosynthesis of stromal matrix proteoglycans and basement membrane components by human corneal fibroblasts. *Invest Ophthalmol Vis Sci*. 1992;33:547–557.
- Funderburgh JL, Mann MM, Funderburgh ML. Keratocyte phenotype mediates proteoglycan structure: a role for fibroblasts in corneal fibrosis. *J Biol Chem*. 2003;278:45629–45637.
- Long CJ, Roth MR, Tasheva ES, Funderburgh M, Smit R, Conrad GW. Fibroblast growth factor-2 promotes keratan sulfate proteoglycan expression by keratocytes in vitro. *J Biol Chem*. 2000;275:13918–13923.
- Chen J, Guerriero E, Sado Y, Sundaraj N. Rho-mediated regulation of TGF-beta1- and FGF-2-induced activation of corneal stromal keratocytes. *Invest Ophthalmol Vis Sci*. 2009;50:3662–3670.
- Jester JV, Petroll WM, Barry PA, Cavanagh HD. Expression of α -smooth muscle (α -SM) actin during corneal stromal wound healing. *Invest Ophthalmol Vis Sci*. 1995;36:809–819.
- Funderburgh J, Funderburgh M, Mann M, Corpuz L, Roth M. Proteoglycan expression during transforming growth factor beta-induced keratocyte-myofibroblast transdifferentiation. *J Biol Chem*. 2001;276:44173–44178.
- Jester JV, Huang J, Petroll WM, Cavanagh HD. TGFbeta induced myofibroblast differentiation of rabbit keratocytes requires synergistic TGFbeta, PDGF and integrin signalling. *Exp Eye Res*. 2002;75:645–657.
- Maltseva O, Folger P, Zekaria D, Petridou S, Masur SK. Fibroblast growth factor reversal of the corneal myofibroblast phenotype. *Invest Ophthalmol Vis Sci*. 2001;42:2490–2495.
- Tomasek JJ, Hay ED, Fujiwara K. Collagen modulates cell shape and cytoskeleton of embryonic corneal and fibroblast fibroblasts: distribution of actin, α -actinin and myosin. *Dev Biol*. 1982;92:107–122.
- Cukierman E, Pankov R, Yamada KM. Cell interactions with three-dimensional matrices. *Curr Opin Cell Biol*. 2002;14:633–639.

27. Grinnell F, Petroll WM. Cell motility and mechanics in three-dimensional collagen matrices. *Annu Rev Cell Dev Biol*. 2010;26:335-361.
28. Chen J, Wong-Chong J, SundarRaj N. FGF-2 and TGF- β 1-induced downregulation of lumican and keratocan in activated corneal keratocytes by JNK signaling pathway. *Invest Ophthalmol Vis Sci*. 2011;52:8957-8964.
29. Lakshman N, Kim A, Petroll WM. Characterization of corneal keratocyte morphology and mechanical activity within 3-D collagen matrices. *Exp Eye Res*. 2010;90:350-359.
30. Kim A, Lakshman N, Karamichos D, Petroll WM. Growth factor regulation of corneal keratocyte differentiation and migration in compressed collagen matrices. *Invest Ophthalmol Vis Sci*. 2010;51:864-875.
31. Kim A, Lakshman N, Petroll WM. Quantitative assessment of local collagen matrix remodeling in 3-D culture: the role of Rho kinase. *Exp Cell Res*. 2006;312:3683-3692.
32. Grinnell F. Fibroblast-collagen matrix contraction: growth-factor signalling and mechanical loading. *Trends Cell Biol*. 2000;10:362-365.
33. Brown RA, Wiseman M, Chu C-B, Cheema U, Nazhat SN. Ultra-rapid engineering of biomimetic materials and tissues: fabrication of nano- and microstructures by plastic compression. *Adv Funct Mater*. 2005;15:1762-1770.
34. Neel EAA, Cheema U, Knowles JC, Brown RA, Nazhat SN. Use of multiple unconfined compression for control of collagen gel scaffold density and mechanical properties. *Soft Matter*. 2006;2:986-992.
35. Jester JV, Barry-Lane PA, Cavanagh HD, Petroll WM. Induction of alpha-smooth muscle actin expression and myofibroblast transformation in cultured corneal keratocytes. *Cornea*. 1996;15:505-516.
36. Wolf K, Alexander S, Schacht V, et al. Collagen-based cell migration models in vitro and in vivo. *Sem Cell Dev Biol*. 2009;20:931-941.
37. Amano M, Ito M, Kimura K, et al. Phosphorylation and activation of myosin by Rho-associated kinase (Rho-kinase). *J Biol Chem*. 1996;271:20246-20249.
38. Parizi M, Howard EW, Tomasek JJ. Regulation of LPA-promoted myofibroblast contraction: role of rho, myosin light chain kinase, and myosin light chain phosphatase. *Exp Cell Res*. 2000;254:210-220.
39. Petroll WM, Ma L, Ly L, Vishwanath M. Analysis of the pattern of sub-cellular force generation by corneal fibroblasts following Rho activation. *Eye Contact Lens*. 2008;34:65-70.
40. Roy P, Petroll WM, Cavanagh HD, Jester JV. Exertion of tractional force requires the coordinated upregulation of cell contractility and adhesion. *Cell Motil Cytoskeleton*. 1999;43:23-34.
41. Vishwanath M, Ma L, Jester JV, Otey CA, Petroll WM. Modulation of corneal fibroblast contractility within fibrillar collagen matrices. *Invest Ophthalmol Vis Sci*. 2003;44:4724-4735.
42. Lee PF, Yeh AT, Bayless KJ. Nonlinear optical microscopy reveals invading endothelial cells anisotropically alter three-dimensional collagen matrices. *Exp Cell Res*. 2009;315:396-410.
43. Bell E, Ivarsson B, Merrill C. Production of a tissue-like structure by contraction of collagen lattices by human fibroblasts of different proliferative potential in vivo. *Proc Natl Acad Sci U S A*. 1979;76:1274-1278.
44. Elsdale T, Bard J. Collagen substrata for studies on cell behavior. *J Cell Biol*. 1972;54:626-637.
45. Grinnell F, Lamke CR. Reorganization of hydrated collagen lattices by human skin fibroblasts. *J Cell Sci*. 1984;66:51-63.
46. Stopak D, Harris AK. Connective tissue morphogenesis by fibroblast traction. *Dev Biol*. 1982;90:383-398.
47. Rhee S, Jiang H, Ho C-H, Grinnell F. Microtubule function in fibroblast spreading is modulated according to the tension state of cell-matrix interactions. *Proc Natl Acad Sci*. 2007;104:5425-5430.
48. Grinnell F, Ho C-H, Tamariz E, Lee DJ, Skuta G. Dendritic fibroblasts in three-dimensional collagen matrices. *Mol Biol Cell*. 2003;14:384-395.
49. Berthaut AV, Mirshahi P, Benabbou N, et al. Insulin growth factor promotes human corneal fibroblast network formation in vitro. *Invest Ophthalmol Vis Sci*. 2011;52:7647-7653.
50. Petroll WM, Ma L, Kim A, Ly L, Vishwanath M. Dynamic assessment of fibroblast mechanical activity during Rac-induced cell spreading in 3-D culture. *J Cell Physiol*. 2008;217:162-171.
51. Tamariz E, Grinnell F. Modulation of fibroblast morphology and adhesion during collagen matrix remodeling. *Mol Biol Cell*. 2002;13:3915-3929.
52. Karamichos D, Lakshman N, Petroll WM. An experimental model for assessing fibroblast migration in 3-D collagen matrices. *Cell Motil Cytoskeleton*. 2009;66:1-9.
53. Andresen JL, Ledet T, Hager H, Josephsen K, Ehlers N. The influence of corneal stromal matrix proteins on the migration of human corneal fibroblasts. *Exp Eye Res*. 2000;71:33-43.
54. Andresen JL, Ledet T, Ehlers N. Keratocyte migration and peptide growth factors: the effect of PDGF, bFGF, EGF, IGF-1, aFGF and TGF-beta on human keratocyte migration in a collagen gel. *Curr Eye Res*. 2007;16:605-613.
55. Pelham RJ, Wang YL. Cell locomotion and focal adhesions are regulated by substrate flexibility. *Proc Natl Acad Sci U S A*. 1997;94:13661-13665.
56. Yeung T, Georges PC, Flanagan LA, et al. Effects of substrate stiffness on cell morphology, cytoskeletal structure, and adhesion. *Cell Motil Cytoskeleton*. 2005;60:24-34.
57. Miron-Mendoza M, Seemann J, Grinnell F. The differential regulation of cell motile activity through matrix stiffness and porosity in three dimensional collagen matrices. *Biomaterials*. 2010;31:6425-6435.
58. Garrett Q, Khaw PT, Blalock TD, Schultz GS, Grotensdorst GR, Daniels JT. Involvement of CTGF in TGF-beta-1-stimulation of myofibroblast differentiation and collagen matrix contraction in the presence of mechanical stress. *Invest Ophthalmol Vis Sci*. 2004;45:1109-1116.
59. Tomasek JJ, Gabbiani G, Hinz B, Chaponnier C, Brown RA. Myofibroblasts and mechanoregulation of connective tissue remodeling. *Nat Rev Mol Cell Biol*. 2002;3:349-363.
60. Meyer-ter-Vehn T, Han H, Grehn F, Schlunck G. Extracellular matrix elasticity modulates TGF- β -induced p38 activation and myofibroblast transdifferentiation in human tenon fibroblasts. *Invest Ophthalmol Vis Sci*. 2011;52:9149-9155.
61. Sabeh F, Shimizu-Hirota R, Weiss SJ. Protease-dependent versus independent cancer cell invasion programs: three-dimensional amoeboid movement revisited. *J Cell Biol*. 2009;185:11-19.
62. Zaman MH, Trapani LM, Siemeski A, et al. Migration of tumor cells in 3D matrices is governed by matrix stiffness along with cell-matrix adhesion and proteolysis. *Proc Natl Acad Sci U S A*. 2006;103:10889-10894.