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MMP Regulation of Corneal Keratocyte Motility and Mechanics in 3-D Collagen Matrices

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Abstract

Previous studies have shown that platelet derived growth factor (PDGF) can stimulate corneal keratocyte spreading and migration within 3-D collagen matrices, without inducing transformation to a contractile, fibroblastic phenotype. The goal of this study was to investigate the role of matrix metalloproteinases (MMPs) in regulating PDGF-induced changes in keratocyte motility and mechanical differentiation. Rabbit corneal keratocytes were isolated and cultured in serum-free media (S-) to maintain their quiescent phenotype. A nested collagen matrix construct was used to assess 3-D cell migration, and a standard collagen matrix model was used to assess cell morphology and cell-mediated matrix contraction. In both cases constructs were cultured in S-supplemented with PDGF, with or without the broad spectrum MMP inhibitors GM6001 or BB-94. After 4 days, f-actin, nuclei and collagen fibrils were imaged using confocal microscopy. To assess sub-cellular mechanical activity (extension and retraction of cell processes), time-lapse DIC imaging was also performed. MT1-MMP expression and MMP-mediated collagen degradation by were also examined. Results demonstrated that neither GM6001 nor BB-94 affected corneal keratocyte viability or proliferation in 3-D culture. PDGF stimulated elongation and migration of corneal keratocytes within type I collagen matrices, without causing a loss of their dendritic morphology or inducing formation of intracellular stress fibers. Treatment with GM6001 and BB-94 inhibited PDGF-induced keratocyte spreading and migration. Relatively low levels of keratocyte-induced matrix contraction were also maintained in PDGF, and the amount of PDGF-induced collagen degradation was similar to that observed in S- controls. The collagen degradation pattern was consistent with membrane-associated MMP activity, and keratocytes showed positive staining for MT1-MMP, albeit weak. Both matrix contraction and collagen degradation were reduced by MMP inhibition. For most outcome measures, the inhibitory effect of BB-94 was significantly greater than that of GM6001. Overall, the data demonstrate for the first time that even under conditions in which low levels of contractility and extracellular matrix

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proteolysis are maintained, MMPs still play an important role in mediating cell spreading and migration within 3-D collagen matrices. This appears to be mediated at least in part by membrane-tethered MMPs, such as MT1-MMP.

Keywords

Cell Mechanics; Matrix Metalloproteinases; Collagen; 3-D Culture; Corneal Keratocytes

1. INTRODUCTION

The corneal stroma, which makes up 90% of corneal thickness, is a highly ordered structure consisting of collagen lamellae with ordered packing and spacing that is critical to maintenance of corneal transparency. Corneal stromal cells (keratocytes) reside between the collagen lamellae, and are responsible for secreting ECM components required to maintain normal corneal structure and function (i.e. transparency). From a mechanical standpoint, resting keratocytes are considered quiescent; they do not express stress fibers or generate substantial contractile forces (Jester et al., 1994; Lakshman et al., 2010). Because it is directly exposed to environmental conditions, the cornea is susceptible to physical and chemical injuries. Because of its accessibility and optical power, it is also the target for numerous refractive surgical procedures, such as photorefractive keratectomy (PRK) and laser-assisted in situ keratomileusis (LASIK).

During wound healing following injury or surgery, quiescent corneal keratocytes generally become activated, and transform into fibroblast and myofibroblast repair phenotypes that mediate cell migration, wound contraction and matrix remodeling (Blalock et al., 2003; Jester et al., 1999a; Jester et al., 1999b; Stramer et al., 2003). Wound healing is also accompanied by changes in expression of both matrix metalloproteinases (MMPs) and tissue inhibitors of metalloproteinases (TIMPs) by corneal fibroblasts (Fini, 1999; Fini and Stramer, 2005; Girard et al., 1993; Kenney et al., 1998; Ye and Azar, 1998). Overexpression of MMPs has been correlated with pathologic conditions such as corneal ulceration, epithelial ingrowth, keratoconus and other complications (Brejchova et al., 2009; Collier, 2001; Fournie et al., 2008; Fournie et al., 2010; Mackiewicz et al., 2006). Overall, the coordinated action of matrix synthesis, degradation and reorganization form an integrated system for repairing and remodeling the corneal ECM. Following refractive surgical procedures such as PRK or LASIK, it is preferable to minimize cellular force generation, fibrosis and matrix remodeling during stromal repopulation, since these processes can alter corneal clarity and refractive power (Dupps and Wilson, 2006; Moller-Pedersen et al., 2000). Such non-disruptive stromal repopulation is also needed following UV cross-linking of the cornea, which is increasingly used as a treatment for keratoconus, since this procedure kills corneal keratocytes in the area of treatment. We have recently demonstrated that quiescent corneal keratocytes cultured in serum-free media can migrate effectively through 3-D collagen matrices when stimulated with PDGF, without expressing stress fibers or generating large contractile forces (Kim et al., 2012). This low-tension migration mechanism could potentially allow cells to repopulate corneal tissue without altering its unique structure, thereby minimizing the loss of corneal transparency. However, it is not

known whether this low-contraction migration is dependent on MMP activation, and associated collagen cleavage or degradation.

Peptide growth factors present in the cornea and tear film, such as IGF, PDGF, FGF, IL-1 α and TGF β , are postulated to play an important role in modulating the keratocyte phenotype during corneal wound healing (Arnold et al., 1993; Kim et al., 1999; Musselmann et al., 2008; Tuominen et al., 2001). In cell culture, these growth factors differentially regulate keratocyte proliferation, cytoskeletal organization, MMP expression and ECM synthesis (Beales et al., 1999; Chen et al., 2009; Etheredge et al., 2009; Funderburgh et al., 2001; Girard et al., 1991; Hao et al., 1999; Jester and Chang, 2003; Jester et al., 2002; Kondo et al., 2008; Li et al., 2001; Long et al., 2000; Lu et al., 2004; Maltseva et al., 2001; Mishima et al., 1998; Parkin et al., 2000; West-Mays et al., 1999). Most previous studies investigating the effect of these growth factors on corneal keratocyte differentiation have been performed using rigid, 2-D substrates. However, keratocytes reside within a complex 3-D extracellular matrix *in vivo*, consisting primarily of type I collagen. 2D surfaces are homogeneous, elastic materials. By contrast, collagen matrices are biphasic, viscoelastic, exhibit non-affine compression (Chandran and Barocas, 2006) and can undergo strain-stiffening (Storm et al., 2005). In short, the mechanical properties of 3-D collagen matrices more closely resemble the connective tissue environment, and significant differences in cell morphology, adhesion organization, and mechanical behavior have been identified in 2-D vs. 3-D culture models (Cukierman et al., 2002; Grinnell and Petroll, 2010; Tomasek et al., 1982). 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. In this study we investigate the dependence of PDGF-induced keratocyte spreading, migration and ECM remodeling on MMPs under serum-free culture conditions, using a variety of 3-D collagen matrix models.

2. MATERIALS AND METHODS

2.1 Materials

Dulbecco's modified Eagle medium (DMEM), MEM non-essential amino acids and 0.25% trypsin/EDTA solution were purchased from Invitrogen (Gaithersburg, MD). Platelet-derived growth factor BB isotype (PDGF), GM6001 (also known as galardin or ilomastat) were obtained from Millipore (Billerica, MA). BB-94 (also known as batimastat) was purchased from Tocris (Bristol, UK). Fetal bovine serum (FBS), fatty acid-free and fraction V bovine serum albumin (BSA), RPMI vitamin mix, HEPES, DMSO, thymidine and Sodium bicarbonate were obtained from Sigma-Aldrich (St. Louis, MO). Penicillin, streptomycin, and amphotericin B were obtained from Lonza inc. (Walkersville, MD). Type I rat tail collagen was purchased from BD Biosciences (Bedford, MA). PureCol® Type I bovine collagen was purchased from Advanced BioMatrix, Inc. (San Diego, CA). Alexa Fluor 488 and Propidium Iodine (PI) were obtained from Molecular Probes, Inc. (Eugene, OR). Collagenase D from *Clostridium histolyticum* and RNase (DNase free) were purchased from Roche (Indianapolis, IN). Rabbit eyes were purchased from Pel Freez (Rogers, AR). Human glu-plasminogen was purchased from Haematologic Tech (Essex Junction, VT).

Glass bottom dishes were purchased from MatTek (Ashland, MA). MT1-MMP mouse-anti-human monoclonal antibody (C9, Santa Cruz, TX)

2.2 Cell Culture

Corneal keratocytes (NRK cells) were harvested from rabbit eyes as previously described (Lakshman and Petroll, 2012). Briefly, to isolate the stromal keratocytes, corneas stripped of both endothelium and epithelium were placed in a solution of collagenase (KGMP) overnight at 37 °C. The keratocytes were dispersed in the solution, centrifuged and re-suspended in serum-free basal media (S-) consisting of DMEM containing pyruvate, supplemented with HEPES, 1% RPMI media, 1% 100× MEM non-essential amino acids, 100µg/mL ascorbic acid, and 1% penicillin/streptomycin/amphotericin B to maintain the keratocyte phenotype (Jester and Chang, 2003). Keratocyte suspensions were seeded into tissue culture flasks in a 37 °C, 5% CO₂ humidified incubator and cultured for up to 7 days before use.

2.3 Cell Proliferation Assay

Hydrated collagen matrices were prepared by mixing acid-resolved, monomeric rat tail Type I collagen with 10× DMEM to achieve a final collagen concentration of 2.5 mg/mL. After adjusting the pH to 7.2 by addition of NaOH, a suspension of cells was immediately mixed with the above collagen solution to achieve a final cell density of 125 cells/µL. A 40 µL aliquot of the cell/collagen mixture was evenly spread over a circular central region on glass bottom dishes. The specimens were then incubated for 30 min at 37 °C to allow polymerization of the collagen, followed by overnight pre-culture in S- media. The dishes were randomly assigned to different experimental conditions (3 samples/condition) and media was changed to S- supplemented with PDGF [50 ng/ml] and either GM6001 [25µM], BB-94 [8µM] or DMSO (vehicle control, same dilution ratio). Dishes were then cultured for an additional 4 days. In some experiments, 2mM thymidine was also added to the media to evaluate its anti-proliferation effect.

Following overnight pre-culture and after 4 days of culture, cells were fixed using 3% paraformaldehyde in phosphate buffer for 10 min and permeabilized with 0.5% Triton X-100 in phosphate buffer for 3 minutes. Propidium iodine (1:100) was then added to each construct to stain the cell nuclei. Cells were then incubated for 15 minutes and washed with PBS (3 times for 5 minutes). After labeling, fluorescent images were acquired using laser confocal microscopy (Leica SP2, Heidelberg, Germany). 3-D image stacks of cell nuclei were acquired by optically scanning throughout the entire thickness of the matrix, in 3–4 randomly chosen 10X fields for each dish. Maximum-intensity projections were used to combine all of the cell nuclei in a 3-D stack into one image. The overall cell density of each culture condition was calculated by counting the number of cells in each projected image.

2.4 Collagen Matrix Dissolution Assay

A collagen matrix dissolution assay (Birkedal-Hansen et al., 2001) was used to test the ability of NRK cells to degrade polymerized rat tail collagen extracellular matrices in response to different growth factors in vitro, and to evaluate the inhibitory function of BB-94 and GM6001 on MMP activities. An aliquot of cell suspension containing 5×10^4

cells was dropped onto the center of a dry, polymerized, fibrillar rat tail Type I collagen film that had been pre-coated on every culture surface of a multiple well plate. The droplet of cell suspension was incubated at 37 °C for 5 hours to ensure cells attached to the collagen substrate. Cells were overlaid with S- media and cultured overnight to allow cell spreading. On the second day, media were switched to S- supplemented with selected growth factors (1mL/well) and either GM6001, BB-94 or DMSO. In some experiments, 20µg/mL human glu-plasminogen was added to the media to activate latent soluble MMPs (Pro-MMPs). The plates were cultured for 7 days, followed by trypsin digestion to remove all of the cells from the collagen films. Coomassie blue staining was then used to visualize the dissolution pattern that the cells produced on the collagen film. Photos of the stained wells were taken using a digital camera.

2.5 Fluorimetric DQ-Collagen Degradation Assay and Live Cell Confocal Imaging

For quantitative assessment of collagen degradation by NRK cells in different culture conditions, a fluorimetric assay based on FITC-conjugated dye-quenched collagen Type I (DQ-collagen) was developed (Wolf et al., 2003). Cold, neutralized, acid-resolved rat tail Type I collagen solution was mixed with DQ-collagen Type I (20:1 v/v) and a NRK cell suspension. 80µL of the cell-collagen mixture was immediately spread over each well (5×10^4 cells/well) of a 12-well plate in an ice bath. The plates were incubated at 37 °C for 50 min for polymerization, followed by overnight culture with 1mL/well of S- media. On the next day, all culture wells were supplemented with or without PDGF [50 ng/ml], and with either DMSO vehicle, BB-94 [8µM], or GM6001 [25µM]. All media were phenol-red free. The positive control for the experiment group was a set of wells with the same DQ-copolymerized collagen substrate, incubated with 1mg/mL purified collagenase from *Clostridium histolyticum*. The negative/background control was no-cell DQ-copolymerized collagen incubated with S- media. Culture supernatant was sampled at 1 and 4 days. FITC fluorescence intensity of the supernatant was measured in a fluorescence microplate reader equipped with standard FITC fluorescent filters (Synergy2, BioTek). Background fluorescence measured from the negative control was subtracted from each measurement. For each condition, the relative fluorescence intensity is the average of 6 experiments.

For 63× laser scanning confocal imaging of the DQ-collagen degradation pattern in the live cell culture, the samples were prepared in the same manner as described above except that cells were cultured on MatTek glass bottom dishes. Following the overnight S- culture, media were supplemented with either: 1) Interleukine-1α w. Human plasminogen; 2) PDGF; 3) 10% FBS; or 4) left unchanged (S- basal control). In some experiments, the cell culture samples were further supplemented with BB-94 [8µM]. Cells were cultured for an additional 4 days. Live cell confocal imaging of the DQ-collagen/cell culture samples was performed on the 4th day of culture, with Life Imaging Services (Basel, Switzerland) temperature + CO₂ control system.

In order to give comparable image data between samples, we used the same excitation laser intensity and offset, the same Z-axis scanning step size, and the same image acquisition parameters. For each culture condition, at least two cell culture samples were scanned to get multiple 63× confocal image Z-stacks. FITC fluorescence images, confocal reflection

images, and DIC images were simultaneously acquired through areas of interest in the 3-D matrices. Z-axis image stacks were maximum-intensity projected to visualize the overall morphologies of cells.

2.6 Membrane Type 1-MMP (MMP-14) staining

S- cultured primary NRK cells were seeded on collagen-coated MatTek dishes and pre-cultured in S- media overnight for cell attaching and spreading. Following the overnight S-culture, media were changed to either Interleukine-1 α w. Human plasminogen or PDGF. Cells were then cultured for an additional 4 days. At the end of culture, cells were fixed in 3% paraformaldehyde for 10 min, blocked by 1% BSA for 1 hour, labeled with MT1-MMP mouse-anti-human monoclonal antibody (1:50) overnight at 4°C, then washed in phosphate buffer saline (PBS; 3 times for 5 minutes) and conjugated with secondary FITC goat-anti-mouse antibody (1:1000) for 1 hour at room temperature. After immunochemical labeling, the samples were imaged using laser scanning confocal microscopy (Leica SP2, Heidelberg, Germany). Fluorescent confocal (for MT1-MMP) and DIC images were obtained simultaneously with a water-immersion 63 $\times$ objective (1.2 NA, 220 μ m free working distance). Z-axis image stacks were maximum-intensity projected to 2D images to display the overall morphologies of cells.

2.7 Global Matrix Contraction Assay

Primary NRK cells were seeded within reconstituted rat tail Type I collagen. 30 μ L of this cell-collagen mixture (containing ~50,000 cells) was spread over a 10 mm diameter circular region on a MatTek dish and then polymerized at 37 °C. Since the bottom of the matrices remain attached to the dish, cell-induced contraction resulted in a decrease in matrix height (Grinnell, 2000). The height of each matrix was measured by focusing on the top and bottom of the matrix at 5 different locations at day 1 and day 4, using a 20 $\times$ DIC imaging as previously described (Lakshman et al., 2010). Measurements were performed in triplicate for each culture condition, and repeated 3 times. The percentage decrease in matrix height over time was then calculated.

2.8 Assessment of Cell Morphology and Local Matrix Reorganization

At the end of each Global Matrix Contraction experiment (4 days), cells were fixed, permeabilized, labeled with Alexa Fluor 488 Phalloidin (1:50) for 1 hour and then washed in phosphate buffer saline (PBS; 3 times for 5 minutes). After labeling, fluorescent (for f-actin) and reflected light (for collagen fibrils) confocal images were acquired simultaneously using a 63 $\times$ objective. A HeNe laser (633nm) was used for reflection imaging, and an Argon (488 nm) laser was used for fluorescent imaging of f-actin.

Maximum intensity projection images of f-actin were then generated in MetaMorph. The images were then imported into Image J and segmented to produce binary images, from which cell outlines were generated. Quantitative morphometric measurements were made from each cell outline using the 'Analyze Particles' module in Image J. For each experimental condition, measurements were taken on 24 single cells randomly sampled from 4 day culture samples.

2.9 Assessment of Dynamic Cell Activity

Cell-seeded collagen matrices were prepared as described for *Global Matrix Contraction Assay*, except with a lower cell density (6000 cells/dish). Live-cell imaging was performed using a Nikon TE300 inverted microscope equipped with an environmental chamber (In Vivo Scientific, MO) (Zhou and Petroll, 2010). 20× differential interference contrast (DIC) images were collected at 20-min intervals for 24 hrs (72 intervals total). To measure the dynamics of cell protrusion and retraction over 24 hrs, a custom circular grid was overlaid on each cell (Figure 1). The grid consisted of 5 rings; each 52 μm (80 pixels) wide. Each ring was divided into an inner and outer region. The number of cell process segments within each region was counted at each time point. The change in the numbers of process segments from one time interval to the next was then calculated for each ring. The cumulative and normalized dynamics of cell activity over 24 hrs was then calculated for each cell using following equations:

$$\text{Cumulative Dynamics} = \sum_{i=1}^5 \sum_{j=1}^{71} |N_{i,j} - N_{i,j+1}| \quad (\text{Equation 1})$$

$$\text{Averaged Total Number of Segments} = (\sum_{j=1}^{72} \sum_{i=1}^5 N_{i,j}) / 72 \quad (\text{Equation 2})$$

$$\text{Normalized Dynamics} = \frac{\text{Cumulative Dynamics}}{\text{Averaged Total Number of Segments}} \quad (\text{Equation 3})$$

i = ring number, j = time interval, $N_{i,j}$ = total number of process segments in ring i at time interval j . For each culture condition, dynamics were calculated using 10 randomly picked cells.

2.10 Cell Invasion Assay

Nested collagen matrices were prepared as outlined in Figure 2. Compressed collagen matrices were first prepared as described previously by Brown and coworkers (Brown et al., 2005). Type I rat tail collagen was diluted using 0.02N acetic acid and 10× DMEM. After drop-wise neutralization with 1N NaOH, a suspension of 8×10^6 keratocytes basal media was added to the collagen mixture. The solution containing cells and 2.5mg/mL collagen was poured into a 3×2×1cm stainless steel mould and allowed to set for 30 minutes at 37°C. Matrices were then compacted by a combination of compression and blotting. A layer of nylon mesh (~50 μm mesh size) was placed on a double layer of absorbent paper. The polymerized matrices were placed on the nylon mesh, covered with a second nylon mesh, and loaded with a 130 g stainless steel block for 5 min at room temperature. This process squeezes media out of the matrix, leading to the formation of a flat, cell/collagen sheet with high mechanical stiffness. To produce the nested matrix, 6 mm “buttons” were then punched from the compressed matrix and placed within acellular uncompressed Type I collagen matrices (Zhou and Petroll, 2010). The outer matrices were made from either rat tail collagen or bovine collagen. The constructs were placed in a humidified incubator for 60 minutes to allow polymerization of the outer matrix. Constructs were then overlaid with 1.5ml of S- media. After overnight pre-incubation, media was replaced with either S- or S-

plus PDGF, supplemented with either GM6001, BB-94 or DMSO. In some experiments, media was also supplemented with 2mM thymidine in order to inhibit cell proliferation.

After 4 days, constructs were labeled with Alexa Fluor 488 Phalloidin and Propidium iodine, as described above. After labeling, fluorescent (for f-actin and nuclei) and reflected light (for collagen fibrils) 3-D confocal image stacks were acquired simultaneously. In order to assess overall cell migration into the outer matrices and the associated subcellular cytoskeleton changes, four regions across the interface of the inner and outer matrix and encasing the migrating front of cells were randomly selected. A 3-D confocal image stack was acquired for each region imaged by changing the position of the focal plane in 5 μ m steps in Z-axis using a 10 $\times$ objective. Maximum intensity projection images of f-actin and PI were generated for each 3-D confocal image stack, and were overlaid with reflection images of the collagen. The 10 $\times$ projection images were used to create a 1.5mm wide montage image for each quadrant. As an index of cell migration, the average number of cells that had migrated out of inner matrix was counted in each quadrant. The distance that cells had traveled was calculated by drawing a straight line between the interface and the leading edge cells – an average of 10 cells was used for each montage.

Live cell time-lapse DIC imaging of migration in the construct was also performed, using the Nikon system described above. At each time interval, a 3-D DIC image stack was collected from the top to the bottom of the construct, at the interface of the inner and outer matrices. Images were collected every 15 minutes for 48 hours. This produced a 4-D dataset which captured the cell migration process from the inner matrix into the outer matrix. Triplicate experiments were performed for each condition. Time-lapse sequences for single planes of interest were extracted from the 4D stack.

2.11 Statistics

All statistical analyses were performed using SigmaStat version 11.0 (Systat Software Inc., CA). Analysis of variance (ANOVA) was used to compare group means. Post-hoc multiple comparisons between groups were performed using the Holm–Sidak method. Differences were considered significant if $P < 0.05$.

3. RESULTS

3.1 MMP Inhibitors do not affect Keratocyte Viability or Proliferation

Consistent with previous studies, NRK cells harvested from corneas and cultured in 3-D collagen matrices in serum free basal media (S-) maintain the quiescent, dendritic keratocyte phenotype observed *in vivo* (Jester et al., 1994; Lakshman et al., 2010). The number of cells in 3-D collagen matrices cultured with PDGF media increased by 69.4% after 4 days of culture, and neither GM6001, BB-94 or DMSO (vehicle) affected keratocyte proliferation in response to PDGF ($p = 0.7583$, one-way ANOVA, blue series in Figure 3). We also examined the anti-proliferation effect of thymidine in our cell culture system. After 4 days of culture, there was no significant change in cell density in any treatment groups. Thus, NRK cell proliferation in response to PDGF was blocked by thymidine, regardless of whether MMP inhibitors were in the media ($p = 0.9535$, one-way ANOVA, red series in Figure 3). In all

experiments, cells maintained a dendritic morphology with no indication of toxicity. Overall, the results suggest that DMSO, GM6001 and BB-94 do not significantly affect NRK cell viability or proliferation in 3-D rat tail type I collagen matrices at the concentrations evaluated.

3.2 Keratocytes in PDGF Produce Low Levels of Matrix Degradation

A collagen matrix dissolution assay was used to test the ability of keratocytes to degrade polymerized fibrillar collagen matrices in response to different growth factors, and also to evaluate the inhibitory effects of BB-94 and GM6001. In this assay, two degradation patterns can be identified. 1) Pericellular, where collagen adjacent to cell membranes is degraded by activated MMPs on the cell membrane (Holmbeck et al., 1999; Sabeh et al., 2009a). In this case collagen degradation is limited to the area where the cell cluster was originally seeded onto the collagen matrix. 2) Distal degradation, which is produced by secretion of soluble collagenolytic MMPs. In this case collagen degradation is observed beyond the edges of the area where the cells have been plated. Plasminogen was used to activate latent soluble MMPs (e.g. pro-MMP1, pro-MMP-8), presumably via the plasminogen-plasmin-proMMP activation cascade (Netzel-Arnett et al., 2002; Wilkins-Port et al., 2009).

IL-1 α has been shown to stimulate both expression and production of MMPs by corneal stromal cells (Kondo et al., 2008; Lu et al., 2004; Lu et al., 2003b; Mishima et al., 1998). In our assay, IL-1 α alone stimulated only minor pericellular collagen degradation by keratocytes in the absence of plasminogen (Figure 4, column 6). In contrast, dissolution of fibrillar collagen matrix in IL-1 α culture was dramatically elevated and expanded beyond the area of the cell cluster in the presence of plasminogen (Figure 4, column 5), suggesting that IL-1 α stimulates expression of soluble pro-collagenases (West-Mays et al., 1995), and these soluble pro-collagenases are subject to plasmin-mediated activation (Hao et al., 1999). Diffusion of these water-soluble, activated collagenases results in collagen degradation in distal areas of the well. Keratocytes in 10% FBS, PDGF, TGF β 1 and S- basal media appeared to produce only pericellular collagen degradation, even though plasminogen was present in each of these conditions. The synthetic MMP inhibitor GM6001 only partially blocked these collagenolytic activities, whereas BB-94 completely blocked collagen dissolution in all conditions studied (Figure 4, rows 2 and 3). It should be noted that FBS contains factors that can block soluble MMP activity, thus any effects of FBS on secreted MMPs may be masked in this assay (Figure 4, column 1). Serum-free media was used for all other conditions (columns 2 – 6).

In order to quantify collagen degradation activities and the efficacy of MMP inhibition in 3-D culture, we developed an assay based on dye-quenched (DQTM) collagen. DQ-collagen can be efficiently digested by reactive MMPs, yielding highly fluorescent peptides that can be visualized by fluorescence microscopy (Sameni et al., 2003), or optically assessed by fluorimetric plate reading devices (Horino et al., 2001; Tsai et al., 2005). Under 63 $\times$ fluorescence confocal live cell microscopy, small clusters of fluorescent spots were associated with NRK cells within 3-D DQ-collagen matrices in S- PDGF culture, which is indicative of on-going pericellular collagen degradation (Figure 5). The fluorescence

intensity of the culture supernatant in our DQ collagen assay was also monitored, using a fluorescent plate reader. The fluorimetric results showed that, in PDGF culture, FITC fluorescence continuously intensified from day 1 to day 4, demonstrating that collagenolytic MMPs produced by NRK cells were acting on the collagen substrate, and releasing fluorescent positive peptides into the media (Figure 5). The supernatant of PDGF vehicle condition had an average fluorescence intensity of 4700 RU on the 4th day of culture - only 6% of the positive control. In the presence of GM6001 or BB-94, the fluorescence intensity was reduced to 72% and 64% of the vehicle, respectively (Figure 5). Hence, it appears that BB-94 blocked the collagenolytic MMPs more effectively than GM6001.

Experiments using only conditioned media from PDGF culture to react with DQ collagen substrate did not show any fluorescence changes over time. In contrast conditioned media from IL-1 α culture induced a 20% increase in DQ collagen fluorescence. These results are consistent with our matrix degradation assay results, which show local, pericellular degradation in response to PDGF, but more widespread degradation in response to IL-1 α .

While matrix degradation was mild and remained pericellular in PDGF, secreted species of MMPs induced strong and extensive collagen matrix degradation in the IL-1 α plus Plasminogen condition, as indicated by disrupted and shortened fibrils in confocal reflection images on the 4th day of culture (Supplemented Figure 1). This observation is consistent with the results of the 2-D matrix dissolution assay (Figure 4).

To investigate the correlation between the collagen degradation activities of NRK cells and membrane type-1 MMP, MT1-MMP expression was evaluated using immunochemical staining. Figure 6 shows representative images of the MT1-MMP staining for PDGF and IL-1 α conditions. Weak but positive MT1-MMP staining was observed in PDGF (6A, B), consistent with the pericellular collagen degradation demonstrated in the matrix dissolution assay (Figure 4). In contrast, the fluorescence signal of MT1-MMP was barely detectable in IL-1 α (6C, D). This is also consistent with the matrix dissolution results (Figure 4-C6). No specific fluorescent signals were observed on cell membranes in the 'no primary antibody' staining control (not shown).

3.3 Cell Invasion is Suppressed by MMP Inhibition

To investigate the role of MMPs in NRK cell migration, a nested collagen matrix model was used. PDGF was used in all experiments to stimulate cell migration (Kim et al., 2012). As shown in Figure 7, when the enzymatic functions of endogenous MMPs were inhibited by GM6001 or BB-94, the ability of NRK cells to invade and migrate into the rat tail collagen matrices was suppressed, resulting in a smaller migration index. A similar pattern of inhibition was found when thymidine was added to the media, thus the differences were not due to changes in cell proliferation. Inhibiting proliferation also reduced variability in the cell invasion data, increasing the power of the statistical tests (Figure 7B), and revealing that the inhibitory efficacy of BB-94 on cell invasion was significantly greater than that of GM6001. The distance NRK cells traveled to the migratory front was also assessed. GM6001 did not impact this distance, whereas BB-94 caused a reduction in the distance by approximately 40% ($P = 0.002$, Figure 7C).

Consistent with previous results, migratory keratocytes in the outer matrices were highly elongated and polarized, and had numerous dendritic processes (Kim et al., 2012). Time-lapse DIC images showed that migrating cells in PDGF repeatedly extended and retracted their long, thin dendritic extensions while moving into the outer matrix (Supplemental Video 1). Tractional forces were generated at the tips of these branching processes as indicated by inward movement of collagen fibrils close to the tips. Dendritic processes generally formed in front of the cell body, progressively elongated, and retracted as the cell body slid past them. A similar pattern of dendritic cell migration was observed in the presence of GM6001 (Supplemental Video 2) and BB-94 (Supplemental Video 3); however, both translocation from the inner to the outer matrix, and subsequent migration speed appeared to be retarded in BB-94. Continuous areas and/or tracks of matrix degradation were not observed. Consistent with DIC imaging results, f-actin labeling showed dendritic morphologies under all three conditions studied, and no stress fibers were observed (Figure 8).

Previous studies have demonstrated that cancer cell migration through rat tail collagen matrices is dependent on MMPs. However, for some types of cancer cells, migration through bovine collagen matrices MMP-independent (Sabe et al., 2009b; Wolf et al., 2003), presumably because of differences in the collagen porosity and cross-linking. In addition to migration experiments in rat tail collagen matrices, we also performed a subset of experiments in which bovine collagen was used for the outer matrices. We found that despite the structural differences between rat tail collagen matrices and bovine collagen matrices, the inhibitory effects of the MMP inhibitors on keratocyte invasion were very similar (Supplemental Figure 2).

3.4 Global Matrix Contraction is Suppressed by MMP Inhibition

A global matrix contraction assay was performed to determine whether endogenous MMPs mediate the contractile activities of PDGF-cultured keratocytes in 3-D matrices. Keratocytes cultured in 10% FBS were also included in the assay, as a high-contractility control (Lakshman and Petroll, 2012). Cells in S- basal media produced only 3.86% and 6.82% matrix contraction after 1 and 4 days, respectively. Culture in 10% FBS transformed NRK cells to a highly contractile fibroblastic phenotype which resulted in the largest contraction percentage among all the experimental groups in this assay (35.8% at 1 day, and 68.5% at 4 days).

3.5 Cell Spreading and Dynamic Mechanical Activity is Altered by MMP Inhibition

We also investigated whether the pattern of PDGF-induced cell spreading in 3-D collagen matrices was impacted by MMP inhibition. Both with and without MMP inhibition, keratocytes formed dendritic processes and did not develop stress fibers (Figure 10). Counts of the total number of dendritic processes for each cell after 4 days of culture did not show significant differences (24.4 ± 9.2 , 27.1 ± 8.3 and 27.3 ± 10.2 for vehicle, GM6001 and BB-94, respectively, $p=0.515$, one-way ANOVA). However, the morphologies of NRK cells among the 3 conditions were significantly different from one another (Figure 10). Specifically, keratocytes in PDGF vehicle condition were more elongated and dendritic than the other two conditions (higher Feret, and lower A/P and Solidity). Keratocytes cultured with GM6001 were smaller and less polarized than the other two conditions (lower

Perimeter and higher Circularity). Keratocytes cultured in BB-94 had larger cell bodies and were more stellar (largest Area and Solidity). These differences can be better appreciated in movies showing 3-D reconstructions over a range of projection angles (Supplemental Videos 4–6).

DIC time lapse imaging of live NRK cells provided additional insights into their dynamic mechanical behavior in collagen matrices. Extension and retraction of cell processes was greatest in the PDGF vehicle condition according to the cumulative dynamics analysis. This activity of cell processes declined with both GM6001 and BB-94, and the decreased was statistically significant with GM6001 (Cumulative dynamics over 24 hrs : 347 ± 93.81 , 251.2 ± 43.44 , 292.4 ± 65.47 , for PDGF vehicle, PDGF GM6001, and PDGF BB94 respectively, $P = 0.016$ between vehicle and GM600). Cells in PDGF vehicle had more processes that extended into the outer rings than cells in PDGF BB94 and PDGF GM6001. Thus we normalized the cumulative activity by the average total number of process segments. Interestingly, there was no significant difference in the protrusive dynamics for a given length of cell process (number of process segments), with or without MMP inhibitors (Normalized dynamics : 9.79 ± 1.20 , 10.02 ± 1.73 , 10.17 ± 1.95 , for PDGF vehicle, PDGF GM6001, and PDGF BB94 respectively, $P = 0.653$). Thus the difference in cumulative cell dynamics is likely due to the reduced length of cell processes following MMP inhibition, which undergo smaller extensions and retractions.

4. DISCUSSION

Following lacerating injury or incisional surgery, contractile force generation by fibroblasts/myofibroblasts is needed to facilitate wound closure and prevent loss of the mechanical integrity of the cornea. However, following refractive surgical procedures such as PRK or LASIK, it is preferable to minimize cellular force generation and fibrosis during stromal repopulation, since these processes can alter corneal shape and transparency (Dupps and Wilson, 2006; Moller-Pedersen et al., 2000). We recently demonstrated that corneal keratocytes can effectively migrate through 3-D collagen matrices using both a high-contraction *mesenchymal* mechanism stimulated by serum, and a low-contraction *dendritiform* mechanism stimulated by PDGF. Low-contraction migration in PDGF was consistently observed in both pepsinized and non-pepsinized matrices, and was not impacted by altering the mechanical constraints of the constructs. A similar mechanism of cell migration was induced when serum-cultured corneal fibroblasts were cultured with Y-27632, which blocks cell contractility and fibroblast and myofibroblast transformation of keratocytes in both 2-D and 3-D culture (Chen et al., 2011; Lakshman and Petroll, 2012). From a clinical standpoint, low-tension *dendritiform* keratocyte migration may facilitate keratocyte repopulation of the stroma following surgery or injury, without alterations in the structural properties of the stromal ECM or increased cellular light scattering that can reduce corneal clarity. In this study we investigated the dependence of PDGF-induced keratocyte spreading, migration and ECM remodeling on MMP activation and collagen degradation, using a unique combination of 3-D culture models.

4.1 Growth factor regulation of MMP expression and proteolytic activity

Fibrotic wound healing is generally accompanied by increased expression of matrix metalloproteinases (MMPs) by corneal fibroblasts (Fini, 1999; Fini and Stramer, 2005; Girard et al., 1993). In the uninjured cornea, proMMP-2 is present in normal corneal epithelium and stroma of rabbits, rats and humans, but is associated with tissue inhibitor of MMP type two (TIMP-2), which blocks its protease activity (Brown et al., 1991). MMP-9, collagenases and stromelysins are not normally detected in the cornea (Girard et al., 1993). Following injury, however, expression of MMP-2, MMP-9 and other MMP species by activated corneal fibroblasts is elevated (Daniels et al., 2003). In cell culture, primary corneal keratocytes in serum-containing media (corneal fibroblasts) express similar MMP species as those observed during corneal wound healing, such as MMP-2, MMP-9, collagenases and stromelysins (Fini et al., 1995; Girard et al., 1991). In vitro studies also have shown that MMP expression by corneal fibroblasts is regulated, in part, by growth factors and cytokines found in the tear film, epithelium and stroma during wound healing, such as interleukine-1 (IL-1) and transforming growth factor- β (TGF- β). IL-1 (α and β), which are secreted by corneal epithelial cells, can significantly increase MMP production and collagen degradation by corneal epithelial and stromal cells (Hao et al., 1999; Kondo et al., 2008; Lu et al., 2004; Lu et al., 2003b; Mishima et al., 1998). TGF- β stimulates MMP-2 and MMP-9 secretion by corneal epithelial cells, but has no significant effect on MMP-2 and MMP-9 expression by corneal fibroblasts or myofibroblasts. It also has an inhibitory effect on collagenase and stromelysin secretion (Girard et al., 1991; West-Mays et al., 1999). PDGF has not been shown to have a significant effect on MMP expression by either corneal epithelial cells or fibroblasts, with the exception of a slight increase of MMP-2 (Li et al., 2001; Parkin et al., 2000).

While MMP-2 and MMP-9 have been well characterized in corneal studies, other MMP species are also expressed in normal and wounded corneas, including membrane-anchored type 1 MMP (MT1-MMP or MMP-14) (Chang et al., 2010; Collier et al., 2000; Onguchi et al., 2009; Smine and Plantner, 1997; Ye et al., 2000). MT1-MMP expression is elevated in wounded corneal epithelium and stroma, and may have a regulatory role in different stages of corneal stroma matrix repair/regeneration. In vitro, MT1-MMP expression by serum-exposed human corneal keratocytes can be elevated by addition of Con A in the culture system, and expression is correlated with the level of proMMP-2 activation (Collier et al., 2000). These findings suggest that MT1-MMP may play a role in regulating the activation of other downstream MMPs (Holopainen et al., 2003) and the turnover and remodeling of ECM.

Our matrix dissolution assay produced results that are generally consistent with those cited above. However, unlike previous studies, we used primary cultures of rabbit corneal keratocytes, without pre-exposure to serum or other factors which can permanently alter growth factor signaling responses (Jester and Chang, 2003). Dissolution of the fibril collagen matrix was dramatically elevated in the presence of IL-1 α and plasminogen, and the degradation pattern extended beyond the area of cell plating. However, in the absence of plasminogen, IL-1 α culture stimulated much lower levels of collagen degradation, which did not extend beyond the cell area. These data suggest that IL-1 α stimulates latent

collagenolytic MMP expression (West-Mays et al., 1995), and that these latent soluble MMPs are subject to plasmin-mediated activation (Hao et al., 1999). Plasmin-mediated activation and diffusion of these water-soluble MMPs resulted in collagen degradation in distal areas of the well. In contrast to IL-1 α , keratocytes in 10%FBS, PDGF, TGF β 1 and S-basal media produced more localized collagen degradation both with and without plasminogen in the media, suggesting recruitment of MT1-MMP in these culture conditions (Holmbeck et al., 1999; Sabeh et al., 2009a). This notion was further supported by MT1-MMP immunochemical staining and the DQ-collagen assay in PDGF culture.

The broad spectrum MMP inhibitors GM6001 and BB-94 both demonstrated inhibitory effects on keratocyte-induced collagen degradation in our study. While GM6001 only partially blocked matrix degradation, results of the matrix dissolution assay and our DQ-collagen fluorimetric assay indicated that pericellular collagen degradation was completely suppressed by BB-94. This is consistent with previous studies, in which BB-94 has been reported to effectively block both secreted and membrane-associated MMPs (such as MT1-MMP), while GM6001 is more specific to secreted types (Martin-Martin et al., 2011; Sabeh et al., 2009a; Sabeh et al., 2004; Townley et al., 2008). Taken together, the data suggest that the pericellular collagen degradation observed in PDGF culture was likely due to membrane-associated MMPs.

4.2 The dependency of PDGF induced migration on MMPs

PDGF stimulates migration of corneal keratocytes within Type I collagen matrices, without causing a loss of their dendritic morphology or inducing formation of intracellular stress fibers. In this study, the ability of NRK cells to invade and migrate into the rat tail Type I collagen matrices was suppressed by BB-94 and GM6001. This is consistent with previous studies using skin or lung fibroblasts (Rowe et al., 2011; Sabeh et al., 2009a) and cancer cells (Zaman et al., 2006), in which BB-94 or similar synthetic MMP inhibitors inhibited most of cell invasion/migration activities in 3D matrices (usually 80~90%). In three-dimensional collagen matrices, proteolytically potent HT-1080 fibrosarcoma and MDA-MB-231 carcinoma cells exhibited a constitutive mesenchymal-type movement including the coclustering of β 1 integrins and MT1-MMP at fiber binding sites and the generation of tube-like proteolytic degradation tracks (Wolf et al., 2003). Such areas of matrix degradation were not detected in our high magnification imaging studies using corneal keratocytes. Thus keratocytes are able to overcome the obstructive barrier of reconstituted collagen matrices and efficiently migrate through the matrix without producing large-scale collagen degradation or matrix remodeling.

Previous studies have shown that in bovine collagen matrices (which have reduced stiffness and increased porosity), HT-1080 exhibited protease-independent migration at near undiminished migration rates (Wolf et al., 2003). Sustained protease-independent migration required transformation to an amoeba-like morphology, cytoskeletal organization and migration mode; i.e. a diffuse cortical distribution of the actin cytoskeleton, formation of constriction rings and propulsive squeezing through preexisting matrix gaps. We also performed a subset of experiments in which bovine collagen was used for the outer matrices (2.5mg/ml, the same concentration as the rat tail collagen). However, we found that despite

the structural differences between rat tail collagen matrices and bovine collagen matrices, the inhibitory effects of the MMP inhibitors on keratocyte migration were very similar, and transformation to an amoeboid migration mode was not observed in either case. Thus corneal keratocytes do not appear to have the same migratory and morphologic plasticity as some cancer cells.

Many common types of fibroblasts and tumor cells utilize MT1-MMP to overcome the steric barrier of the ECM (Sabeh et al., 2009a; Sabeh et al., 2004). Blocking, knockdown or mutation of MT1-MMP can disable the migration of several cell types in rat tail collagen matrices (Sabeh et al., 2004; Zarrabi et al., 2011). MT1-MMP is often clustered in the invasive front of migrating cells, and can colocalize with integrins such as $\alpha v \beta 3$ on the breast carcinoma cell membrane (Deryugina et al., 2001), and CD44, a cell-surface glycoprotein (Mori et al., 2002; Zarrabi et al., 2011). These interactions can result in enhanced cell migration. Another mechanism underlying the migration promoting effect of MT1-MMP in other cell types is the pericellular proteolysis activity conducted by MT1-MMP directly and indirectly via activation of proMMP-2 on the cell membrane (Deryugina et al., 2001; Lu et al., 2003a).

4.3 MMP regulation of PDGF-induced cell spreading and matrix reorganization

Consistent with previous studies, PDGF induced keratocyte elongation through extension of dendritic processes, without formation of intracellular stress fibers, or induction of local ECM compaction and alignment (Lakshman and Petroll, 2012). PDGF also induced much less global matrix contraction than serum. Thus overall, a low-contraction, dendritic phenotype was maintained in PDGF. However, it should be noted that global matrix contraction in PDGF was still greater than that measured in serum-free media. PDGF-induced matrix reorganization was even further reduced by BB-94, suggesting that MMPs play a role in this process.

Evaluation of keratocyte morphology and dynamic activity added insights to the matrix contraction results. When NRK cells are cultured within 3-D collagen matrices, cells extend and retract processes but are generally in a non-migratory state. Tractional forces are generated during extension and retraction, and this can contribute to local matrix reorganization and global matrix contraction (Petroll et al., 2008). We found that MMP inhibition induced significant changes in PDGF-induced cell spreading. Specifically, keratocytes in PDGF vehicle condition were more elongated than cells in either GM6001 or BB-94. Branching of cell processes, which was often observed in the vehicle condition, was also reduced following culture with BB-94. Cumulative cell activity (the frequency and amount of extension and retraction of cell processes) also declined with both GM6001 and BB-94, and reached statistical significance with GM6001. This may explain, in part, the decrease in global matrix contraction. Normalization of the cumulative activity revealed no significant difference in the protrusive dynamics for a given length of process, with or without MMP inhibitors. Thus while cells remain active under MMP inhibition, the formation of long dendritic processes in 3-D collagen matrices is impaired. This may be due to the spatial constraints imposed by the surrounding ECM, and the need for localized degradation to create an unobstructed path for cell extension. Alternatively, MMP regulation

of adhesion receptors and their downstream signaling pathways may also play a role (Bouroulia and Stetler-Stevenson, 2010).

Taken together, the data suggests that even under conditions in which low levels of cell contractility and extracellular matrix proteolysis are maintained, MMPs still play an important role in mediating cell spreading and migration within 3-D collagen matrices. This appears to be mediated primarily by pericellular MMP activity, suggesting a role for membrane-tethered MMPs. Localized matrix degradation may allow keratocytes to navigate through the stroma while limiting harmful MMP activities in other areas of the tissue. Overall, PDGF can stimulate keratocyte proliferation and migration in 3-D ECM without inducing transformation to a contractile phenotype or inducing high levels of ECM proteolysis. PDGF has also been shown to up-regulate synthesis of normal stromal ECM (Etheredge et al., 2009). Thus PDGF may have the potential to contribute to stromal repopulation following injury or surgery without producing significant alterations in corneal shape and transparency.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Highlights

MMP regulation of keratocyte mechanics was assessed using 3-D collagen matrix models

PDGF stimulated keratocyte migration without fibroblastic transformation

PDGF induced low levels of collagen degradation, similar to quiescent keratocytes

Nonetheless, MMP inhibition reduced PDGF-induced keratocyte spreading and migration

This appears to be mediated primarily by localized, pericellular MMP activity

Figure 1.

An example of cell dynamics analysis based on a circular grid. Each colored circular ring has a radial width of 52 microns. To evaluate the length of cell protrusion and retraction more accurately, each colored band was bisected so that a process ≥ 52 microns in length was counted as 2 segments, and a process segment < 52 microns in length was counted as 1 segment. The number of process segments/ring was counted manually at each time point using Image J and logged. The change in the number of segments from one interval to the next was used as an indicator of the frequency and amount of pseudopodial extension and retraction.

Figure 2.
Schematic of procedure used for constructing nested matrix model.

Figure 3.

Cell proliferation in 3-D collagen matrices, with or without thymidine blocking. Cell density is the average of 3 experiments. Initial cell density at Time 0 was 36 ± 3.8 cells/unit area.

Figure 4.

Dissolution patterns produced on fibrillar rat tail collagen matrices by primary NRK cells, following culture with different growth factor/cytokines. **1. Vehicle control** (first row): The areas where cell pellets were seeded had reduced staining, as a result of pericellular collagenolysis. 10% FBS, PDGF, and S- cultures each induced a similar amount of matrix dissolution; whereas TGF β produced less collagen dissolution. IL-1 α and plasminogen induced the strongest collagen degradation, and this extended well beyond the area of cell pellet. **2. GM6001** (second row): Dissolution patterns were weaker when GM6001 was present in culture media, except for IL-1 α + PLG. **3. BB-94** (third row): No dissolution marks were left on the matrices when 8 μ M BB-94 was present in culture media, suggesting that both pericellular and secreted collagen degradation was blocked by BB-94. Images were from one experiment, representing the results of 4 independent experiments.

Figure 5.

Left. Fluorescent spots adjacent to the cell membranes, due to pericellular DQ-collagen degradation by NRK cells (Red). Image shown is maximum projection of a 63× FITC confocal image series (Z-stack) overlaid with the best of focus DIC image. Red signal was contrast enhanced for purposes of the overlay. **Right :** Fluorescence of culture supernatant after 1 and 4 days incubation of keratocytes in DQ-collagen matrices using PDGF containing media (n = 4 independent experiments). All values have been normalized by subtracting the background fluorescence level of no-cell controls measured on the same day, to eliminate the effects of spontaneous DQ collagen degradation. MMP inhibition reduced collagen degradation. Complete digestion of DQ-blended collagen with exogenous collagenase D in no-cell samples raised the fluorescence intensity to ~70,000 units.

Figure 6.

A and C. MT1-MMP immunolabeling. **B and D.** Fluorescent signal overlaid with DIC image to show localization of MT1-MMP labeling. In PDGF, punctate MT1-MMP labeling was detected along cell processes (arrows). Labeling was reduced in the IL-1 α condition.

Figure 7.

Keratocyte migration through 3-D rat tail collagen matrices. (A) 10 $\times$ confocal maximum intensity projection of f-actin organization in the 3-D nested migration model. Images were taken after 4 days of incubation. (B) Cell migration results. Each migration index (cell number/1.5mm region on the interface) is the average of triplicate experiments. All experimental groups had 0.1% of DMSO vehicle in the culture media. In serum free PDGF culture, migration of primary NRK cells in 3-D collagen matrices was suppressed by MMP inhibition, especially when using the broad spectrum MMP inhibitor BB-94. The pattern of

inhibition was similar when cell proliferation was inhibited by using 2mM thymidine (see column series 'PDGF Thy'). (C) Distance from matrix interface to migratory front of NRK cells in outer matrices. BB-94 had a significant inhibitory effect on the distance that NRK cells traveled into the outer matrices.

Figure 8.

Maximum projection overlay of 63× confocal fluorescence and reflection imaging of migratory NRK cells in the nested matrix model after 4 days of culture. *Green*= f-actin organization in migratory NRK cells. *Red* = rat tail collagen fibrils acquired by confocal reflection imaging.

Figure 9.

Percentage of matrix contraction by NRK cells after 1 and 4 days. Matrix contraction percentage for each culture condition was the average of measurements from 3 experiments. Consistent with previous studies (Lakshman and Petroll, 2012), PDGF induced much less matrix contraction than serum. NRK cells cultured in PDGF induced 13.28% matrix contraction over 24 hours, and this was significantly inhibited by BB-94 ($P = 0.035$, Figure 9). Four days of culture in PDGF yielded an average contraction percentage of 24%, which was reduced to 13% by BB-94 ($P = 0.001$). These results suggest that steric blockage of endogenous MMPs by BB-94 can impair the ability of corneal keratocytes to contract extracellular collagen matrices. Interestingly, GM6001 had little effect on global matrix contraction.

Condition	Area	Perim.	A/P Ratio	Feret	Circ.	Solidity
Vehicle	22477.50	5765.70	3.8153	698.45	0.0089	0.1214
SD	8869.29	1705.87	0.9806	195.16	0.0027	0.0408
GM6001	21654.38	4417.97	4.9599	589.61	0.0153	0.1542
SD	6085.96	1292.45	0.8264	139.09	0.0053	0.0340
BB-94	32278.52	6544.94	5.0582	596.65	0.0110	0.1929
SD	11508.13	2038.00	1.2278	103.44	0.0053	0.0519

(value in red = $P < 0.05$, pairwise multiple comparisons using one-way ANOVA)

Figure 10.

Images: F-actin organization of keratocytes cultured in 3-D collagen matrices. Images are the maximum intensity projections of $63\times$ confocal Z-stacks. Cells in PDGF with vehicle generally formed elongated, branching dendritic processes, and had a small cell body. Cells in PDGF w/ BB-94 developed shorter, thinner processes, and had a larger cell body (see supplemental videos 4–6). **Table:** Morphological evaluation from 24 cells, randomly sampled from each condition. NRK cells in PDGF vehicle condition were better spread and more dendritic than the other two conditions. NRK cells in culture with GM6001 were shorter and more circular than the other two conditions. NRK cells under the inhibition of MMPs by BB-94 were less elongated, denser and more stellar than the cells cultured in the vehicle condition. **A/P Ratio** = Area/Perimeter. Generally lower for more dendritic morphologies. **Feret** = Feret's diameter is the longest distance between any two points along the cell boundary, and is used as a measure of cell elongation. **Circ.** = Circularity. A value of 1.0 indicates a perfect circle. As the value approaches 0.0, it indicates an increasingly elongated shape; **Solidity** = [Area of cell]/[Area of the convex hull for cell]. The convex hull can be thought of as a rubber band wrapped tightly around the points that define the selection. Higher values indicate a more compact morphology.