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Individual versus Collective Fibroblast Spreading and Migration: Regulation by Matrix Composition in 3-D Culture

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Abstract

Extracellular matrix (ECM) supplies both physical and chemical signals to cells and provides a substrate through which fibroblasts migrate during wound repair. To directly assess how ECM composition regulates this process, we used a nested 3D matrix model in which cell-populated collagen buttons were embedded in cell-free collagen or fibrin matrices. Time-lapse microscopy was used to record the dynamic pattern of cell migration into the outer matrices, and 3-D confocal imaging was used to assess cell connectivity and cytoskeletal organization. Corneal fibroblasts stimulated with PDGF migrated more rapidly into collagen as compared to fibrin. In addition, the pattern of fibroblast migration into fibrin and collagen ECMs was strikingly different. Corneal fibroblasts migrating into collagen matrices developed dendritic processes and moved independently, whereas cells migrating into fibrin matrices had a more fusiform morphology and formed an interconnected meshwork. A similar pattern was observed when using dermal fibroblasts, suggesting that this response in not unique to corneal cells. We next cultured corneal fibroblasts within and on top of standard collagen and fibrin matrices to assess the impact of ECM composition on the cell spreading response. Similar differences in cell morphology and connectivity were observed – cells remained separated on collagen but coalesced into clusters on fibrin. Cadherin was localized to junctions between interconnected cells, whereas fibronectin was present both between cells and at the tips of extending cell processes. Cells on fibrin matrices also developed more prominent stress fibers than those on collagen matrices. Importantly, these spreading and migration patterns were consistently observed on both rigid and compliant substrates, thus differences in ECM mechanical stiffness were not the underlying cause. Overall, these results demonstrate for the first time that ECM protein composition alone (collagen vs. fibrin) can induce a switch from individual to collective fibroblast spreading and migration in 3-D culture. Similar processes may also influence cell behavior during wound healing, development, tumor invasion and repopulation of engineered tissues.

Keywords

Extracellular Matrix; Fibrin; Collagen; 3-D Culture; Cell Motility

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1. INTRODUCTION

The cornea is an optically clear tissue that forms the front surface of the eye, and accounts for nearly two-thirds of its refractive power. The corneal stroma, which makes up 90% of corneal thickness, is a highly ordered structure consisting of approximately 200 collagen lamellae (Pepose and Ubels, 1992). Corneal stromal cells (keratocytes) reside between the collagen lamellae, and are responsible for secreting ECM components required to maintain normal corneal structure and function (Hassell and Birk, 2010). 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). However, following injury, quiescent corneal keratocytes surrounding the area of injury generally become activated, and transform into a fibroblastic phenotype (Jester et al., 1999b; Stramer et al., 2003). The behavior of these corneal fibroblasts during wound healing has a profound impact on corneal transparency and refractive outcome (Moller-Pedersen et al., 2000; Netto et al., 2005).

Extracellular matrix, ECM, provides both physical and chemical signals that can play important roles in regulating cell mechanical behavior during wound healing (Schultz et al., 2011; Shaw and Martin, 2009) and other biological processes such as development, morphogenesis and cancer (Cox and Erler, 2011; Ghosh and Ingber, 2007; Nelson and Bissell, 2006). Following a full thickness injury in the cornea, a fibrin plug forms in the early stage of wound healing (Jester et al., 1995b; Phan et al., 1989; Zieske, 2001). This plug also contains fibronectin, an adhesive protein that can link cells to other ECM proteins, including fibrin and collagen (Singh et al., 2010). In addition to sealing the anterior chamber, the fibrin plug also provides a substrate through which fibroblasts can migrate. In vivo studies have shown that following full thickness corneal incisions in the rabbit, fibroblasts form an interconnected mesh as they migrate into the wound space, and these interconnections are hypothesized to mediate force transduction during wound contraction (Jester et al., 1995a; Petroll et al., 1993). In contrast, during migration through the collagenous corneal stroma during development or following transcorneal freeze injury, cells assume a spindle shaped morphology, and do not appear to cluster or form an interconnected mesh (Ichijima et al., 1993; Tomasek et al., 1982). Due to the complex nature of the in vivo environment, it is not known whether these two distinct patterns of migration are mediated by differences in the growth factor environment, or differences in the protein composition of the ECM with which the fibroblasts interact (fibrin vs. collagen).

In the current study we directly investigate whether collagen and fibrin differentially regulate the pattern of corneal fibroblast spreading and migration, using a previously described nested matrix model (Karamichos et al., 2009; Kim et al., 2012). We found striking differences in the pattern of corneal fibroblast migration into fibrin and collagen ECM. Fibroblasts migrating into collagen matrices developed dendritic processes and moved individually, whereas cells migrating into fibrin matrices had a fusiform morphology and formed an interconnected network. We also demonstrate that dermal fibroblasts exhibit a similar switch from individual to collective cell migration. Similar differences in cell connectivity were observed when corneal fibroblasts were cultured within or on top of un-nested collagen and fibrin matrices. Overall, these findings suggest that collagen and fibrin differentially modulate the mechanism of fibroblast spreading and migration in 3-D culture. Similar processes may also influence cell behavior during development, tumor invasion and repopulation of engineered tissues.

2. MATERIAL AND METHODS

2.1 Materials

Dulbecco's modified Eagle medium (DMEM) and 0.25% trypsin/EDTA solution were purchased from invitrogen (Gaithersburg, MD). Platelet-derived growth factor BB isotype (PDGF) was obtained from Upstate Biotechnology, Inc. (Lake Placid, NY). Fetal bovine serum (FBS), fatty acid-free and fraction V bovine serum albumin (BSA), RPMI vitamin mix, Hepes, Sodium bicarbonate, Thrombin from human plasma, and monoclonal Anti-Pan Cadherin antibody 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 and plasma fibronectin were purchased from BD Biosciences (Bedford, MA). Fibrinogen (purity > 95%; plasminogen, von Willebrand Factor and fibronectin depleted) was obtained from Enzyme Research Laboratories (South Bend, IN). Alexa Fluor 488 and Propidium Iodine (PI) were obtained from Molecular Probes, Inc. (Eugene, OR). RNase (DNase free) was purchased from Roche (Indianapolis, IN). Anti-fibronectin antibody was obtained from Santa Cruz Biotechnology Inc (Santa Cruz, CA). FITC Goat-anti-rabbit conjugated and Goat-anti-mouse were obtained from Jackson ImmunoResearch (West Grove, PA).

2.2 Cell Culture

Human corneal fibroblasts (HTK cells) (Jester et al., 2003) and dermal fibroblasts immortalized with human telomerase (Miron-Mendoza et al., 2010), were cultured in tissue culture flasks with DMEM containing 10% FBS and supplemented with 1% penicillin, 1% streptomycin, and 1% amphotericin B. Cell culture was carried out at 37°C in a 5% CO_2 humidified incubator. Experiments were carried out using basal media supplemented with 5 mg/ml BSA and 50 ng/ml PDGF. Basal media was composed of DMEM containing pyruvate, supplemented with non-essential amino acids, 1% RPMI vitamin mix and ascorbic acid (Jester et al., 1994). Nested matrix experiments (below) were also carried out using a dermal fibroblasts cell line (a generous gift of Dr. Fred Grinnell) (Miron-Mendoza et al., 2010).

2.3 Nested Cell Migration Model

In order to study 3-D cell migration, cell-populated compressed ECM constructs were nested within acellular uncompressed matrices, using a variation of previously published models (Karamichos et al., 2009; Kim et al., 2010). In this model, a 2 ml neutralized collagen solution was prepared by mixing high concentration rat type I collagen with 0.1 N NaOH, 10X DMEM, and H_2O to achieve a final concentration of 4 mg/ml. Collagen solution was then poured onto a rectangular metal mold (3 cm length, 2 cm width, 1 cm height) and placed in a humidified incubator (37°C, 5% CO2) for 30 minutes for polymerization. During polymerization time, cells were harvested from monolayer cultures with 0.25% trypsin/EDTA and washed with DMEM/10% FBS followed by DMEM. Cells (6×10^6) were mixed with a second 2ml collagen solution, which was added on top of the first collagen layer. After 30 minutes to allow collagen polymerization, the sandwich construct was compressed as previously described (Brown et al., 2005; Karamichos et al., 2009; Kim et al., 2010; Neel et al., 2006). This produced a 200 μm thick construct with an acellular ECM on the bottom and a cell-populated ECM on top. The first collagen layer serves as a spacer that prevents cells from contacting the glass substrate as they migrate out of the matrix, thus ensuring that they interact with the outer fibrin or collagen ECM.

To prepare the nested models, 6 mm diameter buttons were cut with a trephine blade and gently placed on glass bottom culture dishes. Buttons were then covered with a 100 μl solution of collagen or fibrin. Samples were then placed for 30 minutes in a humidified

incubator (37°C, 5% CO_2) to polymerize. After polymerization, migration media was added to each sample. Collagen was prepared by mixing high concentration rat type I collagen with 0.1 N NaOH, 10X DMEM, and H_2O to achieve a final concentration of 2 mg/ml. For fibrin matrices, fibrinogen was warmed for 20 minutes and mixed with DMEM to achieve a final concentration of 1 mg/ml, and this solution was mixed with 0.5 U/ml thrombin to initiate polymerization. Concentrations for collagen and fibrin were selected because they produced similar fibril density and packing, based on confocal reflection imaging. Additional experiments were performed using collagen concentrations of 1mg/ml, 3mg/ml and 4mg/ml.

To prepare outer collagen and fibrin matrices containing fibronectin, collagen and fibrin solutions were mixed with soluble fibronectin in a ratio of 1:10. Subsequently 100 μl of this solution was immediately added to cover the compressed collagen buttons, and allowed to polymerize for 30 minutes prior to adding migration media.

2.4 Standard (Un-nested) Matrix Models

For experiments with cells within collagen and fibrin matrices, 100 μl neutralized solutions of collagen (2 mg/ml) and fibrin (1 mg/ml) containing 20×10^3 cells/matrix were allowed to polymerize for 30 min and then incubated with experimental media containing PDGF. For experiments with cells on the surface of matrices, 100 μl neutralized solutions of collagen (2 mg/ml) and fibrin (1 mg/ml) were allowed to polymerize for 30 min. After matrices polymerized, experimental media with PDGF containing 20×10^3 cells/ml were added to each matrix. For experiments with coated 2-D surfaces, dishes were completely covered with 50 μg/ml neutralized solutions of collagen and fibrin and placed for 1 hour in a humidified incubator (37°C, 5% CO_2). Subsequently dishes were rinsed twice with DMEM serum free media and then migration media with PDGF containing 20^3cells/ml were added to each dish.

2.5 Time-Lapse Imaging

Live-cell imaging of cell migration was performed as previously described (Petroll et al., 2003). Briefly, a Nikon TE300 inverted microscope (TE300; Nikon, Tokyo, Japan) was used. The hardware was controlled using a PC running MetaMorph. To maintain cell viability during imaging, an environmental chamber at 37°C with 5% CO_2 supply was mounted on microscope. A z-stack of images was taken at 10 minutes intervals using a 20X dry objective with Nomarski DIC, or a 10X dry phase contrast objective. Imaging was carried out for 6–24 hours depending on the experimental condition. To create movies of cell movements, a single z-plane image was selected at each time point, so that the same cells were in focus for the entire sequence. MetaMorph software version 7.7 (Molecular Devices Inc.) was used to generate movies of cell movements.

2.6 Immunostaining

Immunostaining was carried out by fixing cells with 3% paraformadehyde in phosphate buffer, PBS, for 10 min, and permeabilizing with 0.5% Triton X-100 in PBS for 15 min. Subsequently samples were washed for 30 minutes with PBS and then incubated with 1% BSA fraction V in PBS. For f-actin labeling, cells were incubated with Alexa Fluor 488 Phalloidin (1:150 ratio) for 60 minutes and then washed for 30 minutes to remove dye excess. For fibronectin (rabbit polyclonal) and cadherin (mouse monoclonal) staining, samples were incubated for 120 minutes with the corresponding antibody in a ratio of 1:100, washed for 60 minutes with PBS, and then incubated for 60 minutes with FITC goat anti-rabbit conjugated antibody (for fibronectin), or FITC goat anti-mouse (for cadherin). Samples were finally washed for 30 minutes to remove excess label. Nuclear staining was carried out after f-actin labeling, by incubating samples for 30 minutes with Propidium

Iodide (1:100 ratio) in PBS containing 1:100 RNase-DNase free. All staining procedures were performed in original culture plates to avoid cell or matrix distortion.

Fluorescence Images were collected with a laser confocal microscope (Leica SP2, Heidelberg, Germany) and reflected light was used to visualize fibrin and collagen fibers. HeNe laser (633nm) was used for reflected light, an Argon laser (488nm) was used for fibronectin and cadherin and a GreNe (543nm) laser was used for f-actin. Images were acquired sequentially to avoid cross talk between fluorescence channels. A stack of optical sections were acquired by changing the position of the focal plane in the z-direction with a step size of 2–5μm using a 20X air objective, or a step size of 1 μm using a 63X water immersion objective (1.2 NA, 220 μm free working distance).

2.7 Imaging Processing and 3D Reconstruction

Imaging processing, quantitative analysis of migrating cells and 3D reconstruction of z planes were carried out using MetaMorph software. Images taken in each z plane were combined into one single picture using the maximum intensity projection function. The amount of cell migration was determined by counting the number of cells that migrated into the outer matrix. Counts were averaged from four 20X microscopic fields in each construct, selected arbitrarily. Migrating cells were detected by nuclear staining with Propidium Iodide. Each field included the border of the button and the furthest moving cell. Duplicate samples were analyzed for each condition in each experiment. Final results for each condition are the mean and standard deviation of three separate experiments. For 3D reconstruction of cell migration, z-stacks of images were collected from the first migrating cell visualized on the top of the outer matrix to the last cell visualized in the bottom. The 3D reconstruction function was used in Metamorph to create movies of maximum intensity projections over a range of 3D angles.

2.8 Rheometry

The stiffness of collagen and fibrin matrices was measured by oscillation rheometry using an AR-G2 rheometer (TA instruments, New Castle, DE) with parallel plate geometry. 12 mm diameter collagen and fibrin samples were polymerized in special designed plates that were later mounted to the base plate of the rheometer. Subsequently a 12 mm diameter plate was lowered onto the sample until filling the gap between both plates. Measurements were carried out in a controlled 21°C room temperature. For initial characterization of matrix response, the shear modulus was measured using an oscillatory stress sweep at a frequency of 0.5 Hz until sample failure. Once the linear response of matrices was found, subsequent measurements were performed with an oscillating fixed 3 μNm torque at a frequency of 0.5 Hz for 20 min. Storage (G') modulus was recorded for each sample. Data presented for each condition are based on measurement of three samples in two separate experiments.

3. RESULTS

3.1 Cells Can Migrate Individually in Collagen, but Remain Interconnected in Fibrin

In the nested migration model, cell-populated buttons are embedded in cell-free collagen or fibrin matrices. Cells migrate out of the inner button and into the outer matrix. We compared corneal fibroblast migration into collagen and fibrin outer matrices using media containing PDGF. PDGF is endogenously expressed in corneal tear fluid, and has been shown to increase Rac activity and induce cell spreading and migration in both dermal and corneal fibroblasts (Grinnell, 2000; Petroll et al., 2008). Consistent with previous studies (Karamichos et al., 2009; Kim et al., 2010), corneal fibroblasts stimulated with PDGF assumed an elongated morphology with dendritic processes at the leading edge when migrating into collagen ECM (Figure 1A). Fibroblasts generally migrated into collagen

ECM as “individuals” that became separated from neighboring cells; this is best appreciated from time-lapse movies (Supplemental Video 1). As time progressed and the number of cells in the outer matrix increased, interconnections between cells were observed, but these were generally limited to the tips of dendritic processes and were usually transient in nature. 3D reconstructions show that cells also migrated at different Z angles, and did not coalesce into a single plane during migration (Supplemental Video 2).

When fibrin was used as the outer matrix, the rate of cell migration was significantly reduced (Figure 1I). Importantly, there was also a striking difference in the pattern of cell movement observed in our nested model (Figure 1B). Unlike the individual cell migration observed in collagen ECM, invasion into fibrin was carried out by interconnected groups of cells extending from the inner button (Supplemental Video 3), and isolated cells were not observed. 3D reconstructions show that cells tended to coalesce into a single plane and form an interconnected meshwork as migration progressed (Supplemental Video 4). A recent study using dermal fibroblasts also demonstrated a reduction in the migration rate in fibrin as compared to collagen (Hakkinen et al., 2011); however differences in cell connectivity were not assessed, since the focus was on isolated cells plated at very low density. When we used dermal fibroblasts in our nested matrix model, similar differences in cell connectivity were observed between collagen and fibrin matrices (Figure 2, Supplemental Videos 5 and 6).

Cells initially migrated into fibrin matrices by axon-like extension of their leading edge, while maintaining connection to cells in the inner matrix at the rear. Other cells followed behind the leading cells along the same paths, producing long lines of interconnected cells (Figure 1C). When cells divided, they temporarily detached from other cells, then moved back along the same path to reattach (Supplemental Video 7). Prior to this reattachment, visible tracks were often observed in the fibrin ECM between cells. These observations suggest that tracks or conduits are created by the initial front of migrating cells, and cells behind them prefer to stay within these tracks, even if they become temporarily separated. As migration proceeded, cells having lateral protrusions often became connected to neighboring cells, producing an interconnected mesh. Migrating cells in fibrin were also broader and expressed more stress fibers than cells in collagen matrices (Figure 1B and 1D). To visualize cell-induced matrix reorganization, confocal reflection imaging was used (Lakshman et al., 2010). Cells reorganized matrix fibers in both conditions, as indicated by alignment of ECM fibrils surrounding cells, particularly at the leading edge of the cell processes (Fig. 1E and F, arrows).

3.2 Fibronectin Increases Cell Migration into both Collagen and Fibrin

It has been reported that fibronectin is necessary for cells to migrate (Greiling and Clark, 1997b), or that it plays a role increasing cell migration (Andresen et al., 2000b). In order to evaluate if fibronectin could increase cell migration in our model, fibronectin was added to the outer collagen and fibrin matrices. Addition of fibronectin increased the amount cell migration rate into both collagen and fibronectin matrices (Fig. 1I). However, addition of fibronectin did not change the patterns of migration (Fig. 1G and 1H). Cells still migrated primarily as individuals in collagen-fibronectin matrices, but were interconnected in fibrin-fibronectin matrices (Supplemental Video 8). Clusters of interconnected cells expressing stress fibers often formed in fibrin-fibronectin as migration proceeded (Supplemental Figure 2).

3.3. Cells Within and On Top of Standard Fibrin Matrices also Cluster and Interconnect

In order to determine whether the differences in migratory pattern were unique to our nested matrix model, we also cultured cells inside standard collagen and fibrin matrices and

evaluated their interactions during spreading and migration. Figure 3 shows DIC images from time-lapse videos of cells within fibrin and collagen matrices. Cells were initially rounded and dispersed in both matrix types. After 9 hours of incubation, cells in collagen matrices were elongated, and repeated extension and retraction of cell processes was observed (Supplemental Video 9). Although cells interacted with each other via dendritic processes, these interactions were generally transient, and cells often migrated away from each other after making contact (compare positions of cells 1–4 at beginning and end of sequence). Clusters of cells did not form even after 18 hours of culture. On the other hand, cells in fibrin matrices did not elongate or spread as rapidly, and most cells remained somewhat rounded even after 9 hours of culture (Figure 3). As cells did spread and make contact with each other, they immediately formed broad and stable interconnections (Supplemental Video 10). This led to the formation of cell clusters by 18 hours (compare positions of cells 1, 3 and 4 at beginning and end of sequence). These data show that corneal fibroblasts inside standard matrices also become interconnected in fibrin, and behave more independently in collagen.

In order to evaluate the effects of the 3D environment on these differences in cell behavior, we plated cells on top of collagen and fibrin matrices. Cells in this 2D environment spread faster than observed in 3D, therefore only the first 6 hours of spreading and migration were analyzed. Corneal fiboblasts spread more rapidly on collagen matrices than on fibrin matrices (Fig. 4A,B). Furthermore, cells on collagen matrices developed a thin dendritic morphology (Fig. 4C), as reported previously by other labs (Jiang et al., 2008; Miron-Mendoza et al., 2010), while cells on fibrin assumed a spread, flattened morphology (Fig. 4D). Time lapse imaging demonstrated that cells on collagen interacted with each other transiently via their dendritic processes, but rarely formed stable interconnections or clusters (Supplemental Video 11). In the case of fibrin matrices, as cells spread and made contact with each other, they immediately coalesced and formed broad and stable interconnections (Supplemental Video 12). This led to the formation of cell clusters by 6 hours (Fig. 4E, F). Together our data show that in both 2-D and 3-D environments, cells interacting with collagen move individually, while cells interacting with fibrin form clusters.

3.4 Cadherin is Present between Interconnected Cells

Cadherins are transmembrane proteins found in cell-cell adhesions during collective migration (Ilina and Friedl, 2009). To evaluate if cadherin proteins were present in corneal fibroblast clusters, cells were cultured on fibrin matrices overnight, then fixed and stained for f-actin and cadherin. As shown in Figure 5A, cadherin was present between interconnected cells (arrows), suggesting it is involved in mediating cell-cell adhesion.

3.5 Fibronectin is Secreted by Cells on Fibrin ECM

Fibronectin is an adhesive protein that binds cells to other matrix components such as collagen and fibrin (Singh et al., 2010). Since fibroblasts do not bind well to fibrin, fibronectin may mediate attachment of cells to fibrin and thereby promote cell migration (Andresen et al., 2000b). In order to evaluate the distribution of fibronectin, cells were cultured overnight on fibrin matrices, and labeled for f-actin and fibronectin. Fibronectin was located between cells in the clusters that formed on fibrin, and was often concentrated at the tips of cell processes extending at the edge of the cluster (Figure 5B). Thus fibronectin may play a role in mediating cell attachment to the fibrin, and/or stabilizing or forming the cell-cell attachments within the cluster.

3.6 The Role of ECM Stiffness on Fibroblast Spreading and Migration Patterns

Concentrations for collagen (2mg/ml) and fibrin (1 mg/ml) were selected because they produced similar fibril density and packing, based on confocal reflection imaging. In order

to determine whether the stiffness of these matrices was different, rheometry was performed. Rheometry demonstrated that the elastic (storage) modulus of 1 mg/ml fibrin matrices was higher than that of 2 mg/ml collagen matrices (67 Pascals vs. 8.5 Pascals). We also tested 4 mg/ml collagen matrices, and found the stiffness to be similar to fibrin (Figure 6A). To determine whether the differences in cell morphology and connectivity between fibrin and collagen were due to differences in matrix stiffness, we studied cell migration using a range of outer matrix collagen concentrations. The cell migration number was similar for all collagen concentrations tested (Figure 6B), as was the cell morphology and patterning. We also studied cell spreading on collagen- and fibrin-coated glass surfaces, which should have much higher stiffness. Cells on collagen-coated rigid surfaces continued to develop dendritic processes and remained separated in most areas. In some areas with higher cell density, interconnections between dendritic processes were observed, but cell clustering never occurred (Figure 6C). In contrast cells on fibrin-coated surfaces developed broad interconnections which consistently led to cluster formation (Figure 6D). Thus overall, matrix composition appears to be the primary factor inducing differences in cell clusteri – not matrix stiffness.

4. DISCUSSION

In this study, we used a nested matrix construct to directly compare the pattern of keratocyte migration into collagen and fibrin matrices under controlled 3D culture conditions. Corneal fibroblasts generally migrated into collagen ECM as “individuals” that broke free of cells in the inner matrix and also remained separated from neighboring cells, as previously reported for dermal fibroblasts using a similar model (Miron-Mendoza et al., 2008). As time progressed and the number of cells in the outer matrix increased, interconnections between cells were observed, but these were generally transient in nature. In contrast, cells migrating into fibrin matrices extended their leading edge into the fibrin while maintaining connection to cells in the inner matrix at the rear. Other cells followed behind these cells along the same paths, producing long lines of interconnected cells. This pattern of collective cell migration has been termed “multicellular streaming” (Friedl and Wolf, 2009), which is thought to play a role in directional guidance of neural crest cells during embryonic development (Davis and Trinkaus, 1981; Kasemeier-Kulesa et al., 2005; Teddy and Kulesa, 2004). As migration into fibrin proceeded, adjacent cells having lateral protrusions become interconnected, resulting in the formation of a mesh-like structure. This migration pattern differs from the forward movement of an interconnected cell sheet characteristic of epithelial cells (Farooqui and Fenteany, 2005). It is also somewhat different than the classical “collective” cell migration pattern used by cancer cells, in which lateral connections between invading cell clusters are not typically observed (Ilina and Friedl, 2009). Interestingly, in vivo studies have shown that following full thickness corneal incisions, fibroblasts form an interconnected mesh as they migrate into the wound space, and these interconnections are hypothesized to mediate force transduction during wound contraction (Jester et al., 1995a; Petroll et al., 1993). Importantly, we observed a similar pattern of interconnected migration into fibrin when using dermal fibroblasts, suggesting that his behavior is not unique to corneal cells.

Following cell division in collagen ECM, cells always migrated in opposite directions and did not re-attach to one another. However, when cells in fibrin divided, they detached from each other only temporarily, then quickly moved back along the same paths to reattach. Similar differences were observed following division when cells were plated on top of collagen or fibrin matrices. Interestingly, following cell division inside fibrin ECM, visible tracks were sometimes observed in the fibrin ECM between cells by DIC imaging, and cells remained within these tracks when reattaching. These observations suggest that tracks or conduits are created by the initial front of migrating cells, and cells behind them prefer to stay within these conduits, even if they become temporarily separated. Microtracks of

aligned collagen been identified previously during cell migration in 3D matrices, and they seem to be necessary for the transition from individual to collective cell migration in cancer cell invasion (Wolf et al., 2007).

Fibronectin is an adhesive protein secreted by cells that facilitates binding to other ECM proteins (Singh et al., 2010). In skin tissues, both fibrin and fibronectin are found in the fibrin clot, the provisional matrix following injury (Clark, 1996). In vivo corneal wounds also have high levels of fibronectin (Jester et al., 1995b). Fibronectin synthesis has also been shown to contribute to endothelial cell network formation and tubulogenesis in vitro (Reinhart-King, 2011; Zhou et al., 2008). In our model, fibronectin was secreted within the cell clusters that formed on fibrin ECM. Fibronectin was most concentrated at the tips of cell processes extending from the edge of these clusters, suggesting fibronectin may play a role in mediating cell attachment to the fibrin. Since most cells require fibronectin in order to bind to fibrinogen and fibrin (Greiling and Clark, 1997a; Grinnell et al., 1980), leading edge cells might secrete fibronectin to facilitate binding to the fibrin ECM during migration, and cells behind them likely prefer to stay within these fibronectin conduits, resulting in interconnected lines of cells. When fibronectin was added to the outer collagen and fibrin matrices in the current study, the cell migration rate increased in both cases, consistent with previous results (Andresen et al., 2000a). However, the pattern of migration did not change, suggesting that exogenous fibronectin does not replace the function of localized, cell-secreted fibronectin.

A hallmark of collective cell migration is the presence of cell-cell junctions (Ilina and Friedl, 2009). In collective cell migration, the rear of the front cell remains coupled to the cell behind it during migration (Ilina and Friedl, 2009). Cell-cell adhesion is mainly mediated by cadherins. Consistent with this result, we observed the presence of cadherin in regions of cell-cell interaction on fibrin ECM. Fibronectin may also play a role in mediating cell-cell adhesion, by serving as a bridge for integrin-integrin interactions between cells (Ilina and Friedl, 2009). For example, α5β1 integrin binds to fibronectin along interfaces between ovarian carcinoma cells (Casey et al., 2001) or fibroblasts (Salmenpera et al., 2008), and blocking of β1-integrin function in migrating multicellular melanoma clusters leads to loss of cell-cell cohesion, cell detachment and transition to single-cell migration (Hegerfeldt et al., 2002). We observed high levels of secreted fibronectin around and between cells that had formed clusters on fibrin matrices, suggesting a possible role in cell adhesion. Previous studies have shown that fibronectin binding is required for keratocyte transformation to a myofibroblast phenotype (Jester et al., 1999a). Consistent with these observations, cells interacting with fibrin matrices had a broader morphology and expressed more stress fibers than cells in collagen matrices. Experiments blocking α5 binding or fibronectin secretion are needed to bring more insight into the role of fibronectin in mediating both cell-cell and cell-matrix interactions in fibrin matrices.

3D ECMs are composed of a network of hydrated fibers that have the capacity to undergo reorganization (Chandran and Barocas, 2006). Cell migration and cell-matrix interactions are affected by both the elasticity of the ECM, and the geometrical organization of the fiber network, i.e. the density of the fibers and the space between fibers (Alexander et al., 2008; Miron-Mendoza et al., 2010; Zaman et al., 2006). For this study, we selected concentrations of collagen (2mg/ml) and fibrin (1 mg/ml) that produced similar densities of fibers, as visualized by confocal reflection imaging. However, rheometry measurements indicated that the fibrin matrices were much stiffer. To investigate whether differences in stiffness impacted fibroblast morphology and connectivity during migration, we performed additional experiments using 4mg/ml collagen, which had a similar stiffness to the 1mg/ml fibrin matrices. We also studied cell spreading on collagen- and fibrin-coated glass surfaces, which have very high stiffness as compared to hydrated 3-D matrices. In all cases, cells interacting

with collagen assumed a dendritic morphology and continued to spread and migrate individually, whereas cells interacting with fibrin interconnected and formed clusters. Previous studies have shown that vascular endothelial cells tend to aggregate on compliant substrates, and remain separated on stiffer substrates (Reinhart-King, 2011). Furthermore, dermal fibroblasts on collagen matrices can form aggregates when they are in a pro-contractile growth factor environment (serum, LPA), but the amount of clustering is inversely related to matrix stiffness (Rhee et al., 2010). Thus based on matrix elasticity alone, one would expect less cell aggregation in fibrin ECM. Overall, the data suggest that differences in ECM stiffness are not the underlying cause for the difference in cell connectivity observed between collagen and fibrin matrices.

In conclusion, our results demonstrate for the first time that ECM composition (collagen vs. fibrin) can play an important role in modulating the pattern of fibroblast spreading and migration in 3-D culture. This may explain, in part, the different migratory patterns observed during corneal wound repair *in vivo*. Similar processes may also influence cell behavior during development, tumor invasion and repopulation of bioengineered tissues.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Highlights

- Corneal fibroblasts migrating into collagen move independently.
- Corneal fibroblasts migrating into fibrin form an interconnected meshwork.
- Dermal fibroblasts migrating into collagen and fibrin exhibit similar differences in cell behavior.
- Fibroblasts cluster within or on top of standard fibrin matrices, but not on collagen matrices.
- These differences are consistently observed on both rigid and compliant ECM environments.

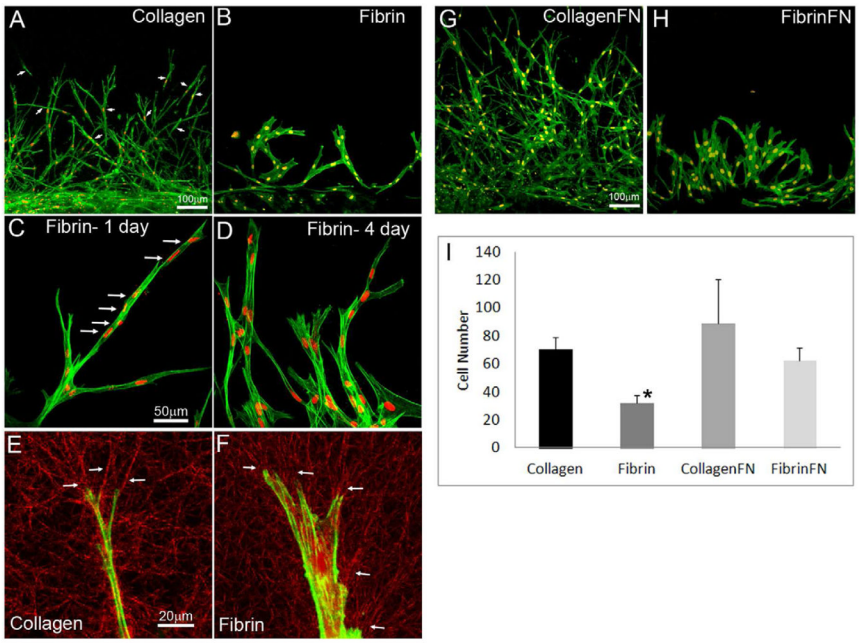

Figure 1.

Cell migration in nested collagen and fibrin matrices. (A, B) Overlays of f-actin and nuclear labeling showing cell migration from inner button into outside matrix after 48 hours of incubation. Outer matrix was either collagen (A) or fibrin (B). (C, D) Overlays of f-actin and nuclear labeling showing cell migration into fibrin after 1 day (C) or 4 days (D) of incubation. (E, F) Fluorescence and reflection microscopy of single migrating cells in collagen (E) and fibrin (F) matrix. Fiber alignment at the leading edge (arrows) shows that cells pull and reorganize matrix during migration. (G, H) Overlays of f-actin and nuclear labeling showing cell migration from inner button into outside matrix after 48 hours of incubation, with the addition of fibronectin to the outer collagen (G) and fibrin (H) matrices. (I) Graph showing cell counts in the outer matrix after 48 hours of incubation. Results are mean +/− SD based on three separate experiments with duplicate matrices at each matrix condition. (* $P < 0.05$ as compared to collagen and collagen-FN, ANOVA)

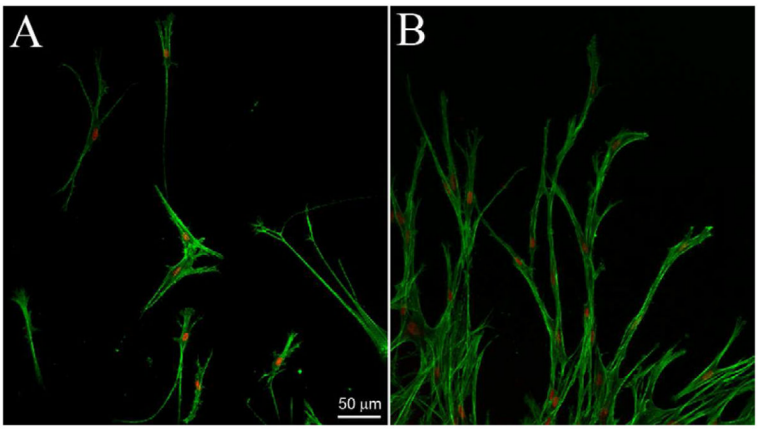

Figure 2.

Overlays of f-actin and nuclear labeling of dermal fibroblasts migrating into collagen (A) or fibrin (B) matrix after 3 days of incubation. The leading edge of migratory fronts are shown. Note that cells orientate at different angles and can migrate individually in collagen matrices; whereas invasion into fibrin was carried out by long interconnected groups of cells extending from the inner buttons.

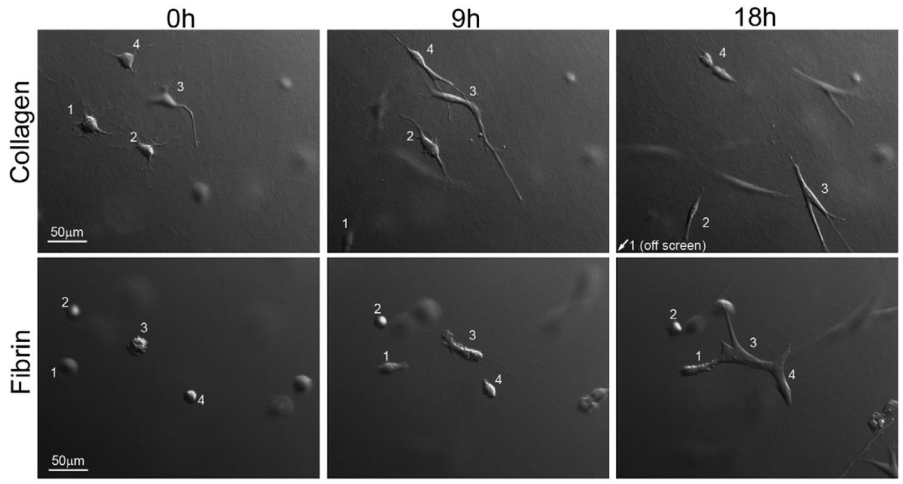

Figure 3.

Motile activity of corneal fibroblasts within collagen and fibrin matrices. DIC images from time-lapse videos of fibroblasts incubated for 18 h within collagen or fibrin matrices. Cells within collagen matrices moved individually, while cells within fibrin matrices grouped together to form clusters.

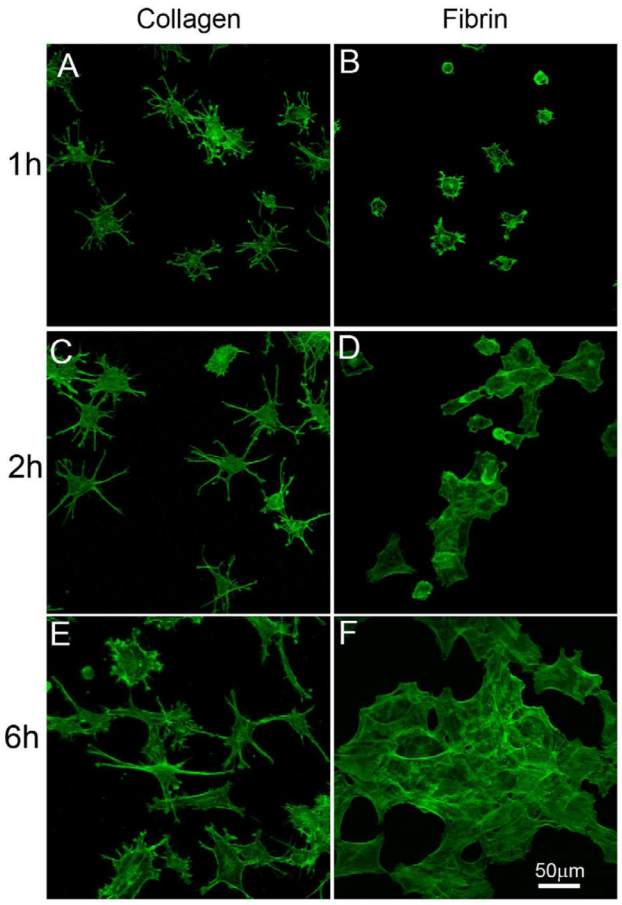

Figure 4.
Spreading pattern of corneal fibroblasts on top of collagen and fibrin matrices. F-actin labeling after incubation for 6 hours on top of collagen or fibrin matrices. Corneal fibroblasts on collagen matrices spread rapidly and developed a dendritic morphology, while cells on top of fibrin matrices grouped together to form clusters.

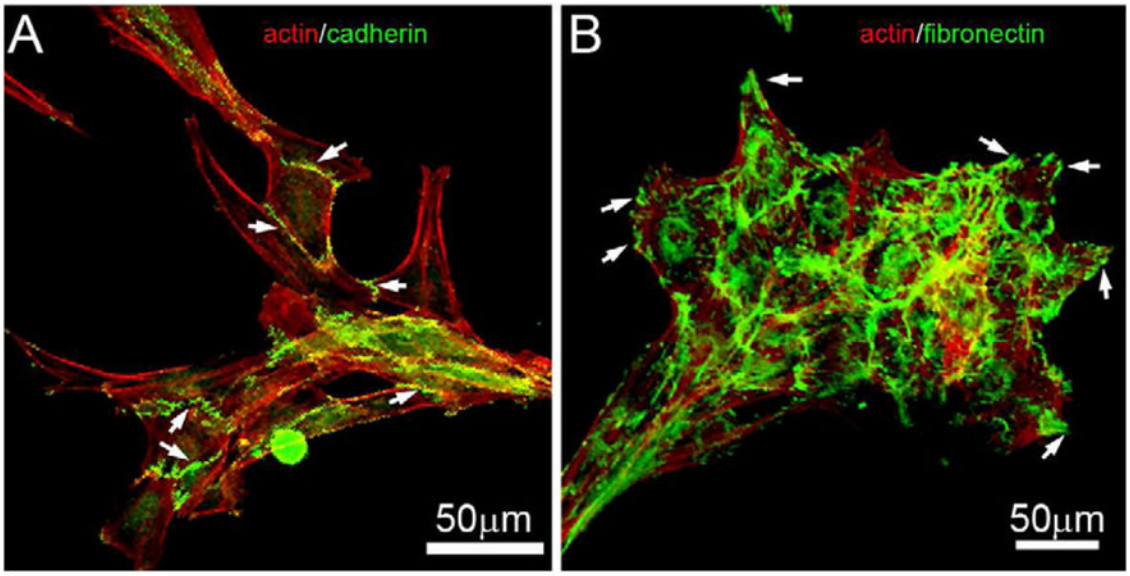

Figure 5.

Cadherin and fibronectin organization for cells on fibrin matrices. Corneal fibroblasts were incubated overnight (18 h) on top of 1 mg/ml fibrin matrices. At the end of the incubation, cells were labeled for f-actin and cadherin (A), or f-actin and fibronectin (B). Cadherin was localized between interconnected cells (A, arrows). Fibronectin was located between cells in the clusters that formed on fibrin. In addition, fibronectin was often concentrated at the tips of cell processes extending at the edge of the cluster (B, arrows).

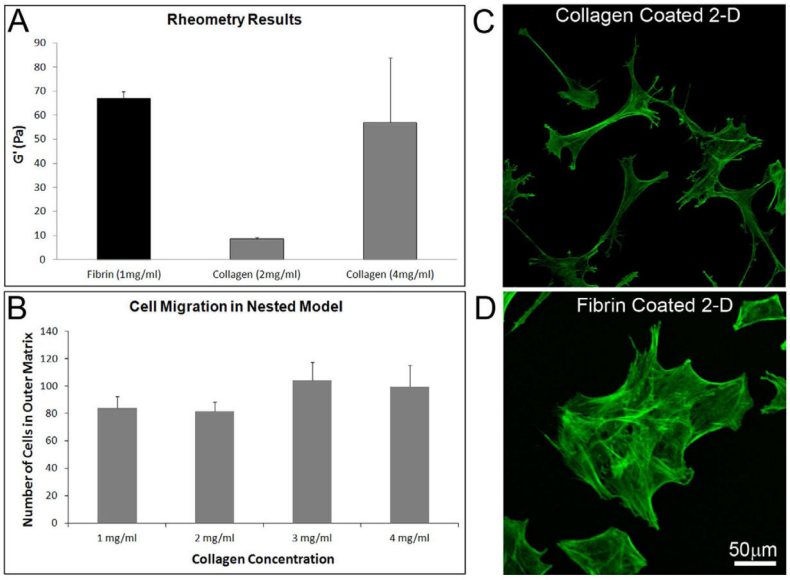

Figure 6.

Role of ECM stiffness on cell connectivity. (A) Matrix Stiffness was measured using an AR-G2 rheometer with parallel plate geometry. The elastic (storage) modulus of 1 mg/ml fibrin matrix was higher than that of 2 mg/ml collagen matrix, but was similar to that of 4 mg/ml collagen matrix. Pa = Pascals. (B) Collagen matrix concentration did not play a significant role in cell migration. Cell migration number and pattern was similar for all concentrations tested (P=0.153, ANOVA). Results are mean +/− SD based on three separate experiments with duplicate matrices at each matrix condition. (C and D) Collagen and fibrin coated surfaces (which should both have the same high stiffness), also showed the same differences in cell behavior as hydrated matrices. On collagen-coated surfaces, dendritic processes sometimes interconnected in areas of high cell density, but cell clustering was not observed. In contrast cells on fibrin-coated surfaces consistently grouped together and formed clusters.