Collagen Fibril Flow and Tissue Translocation Coupled to Fibroblast Migration in 3D Collagen Matrices

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In nested collagen matrices, human fibroblasts migrate from cell-containing dermal equivalents into surrounding cell-free outer matrices. Time-lapse microscopy showed that in addition to cell migration, collagen fibril flow occurred in the outer matrix toward the interface with the dermal equivalent. Features of this flow suggested that it depends on the same cell motile machinery that normally results in cell migration. Collagen fibril flow was capable of producing large-scale tissue translocation as shown by closure of a ~1-mm gap between paired dermal equivalents in floating, nested collagen matrices. Our findings demonstrate that when fibroblasts interact with collagen matrices, tractional force exerted by the cells can couple to matrix translocation as well as to cell migration.

INTRODUCTION

Cell migration depends on the multistep process of cell extension, adhesion, exertion of backward tractional force, and tail retraction (Lauffenburger and Horwitz, 1996; Mitchison and Cramer, 1996; Galbraith and Sheetz, 1997; Beningo et al., 2001; Ridley et al., 2003). The extensive body of research underlying the multistep model is based on studies with tissue cells on extracellular matrix (ECM)-coated planar surfaces including rigid materials such as glass or plastic coverslips as well as flexible materials such as polyacrylamide. On ECM-coated planar surfaces, cells can modulate their cytoskeletal function and adhesion strength in response to surface mechanics (Balaban et al., 2001; Discher et al., 2005; Ingber, 2006; Vogel and Sheetz, 2006). However, adsorbed or covalently attached ECM molecules tend to be held in register with each other with little capacity to undergo cell-mediated mechanical and molecular reorganization.

Type I collagen is the major protein component of fibrous connective tissues. These connective tissues provide mechanical support and frameworks throughout the body, and fibroblasts are the cell type primarily responsible for their biosynthesis and remodeling. Three-dimensional (3D) matrices prepared with type I collagen exhibit mechanical properties that resemble connective tissue (Barocas et al., 1995; Wakatsuki et al., 2000; Roeder et al., 2002; Silver et al., 2002; Ahlfors and Billiar, 2007). Unlike ECM-coated material surfaces, fibroblasts can mechanically remodel collagen matrices both locally and globally (Brown et al., 1998; Tomasek et al., 2002; Grinnell, 2003; Petroll, 2004; Tranquillo, 1999) Such mechanical remodeling of connective tissue ECM is believed to be important for tissue homeostasis (Silver et al., 2002; Wiig et al., 2003; Goldsmith et al., 2004; Langevin et al., 2004), aging (Varani et al., 2004), repair (Tonnesen et al., 2000; Tomasek et al., 2002; Grinnell, 2003), fibrosis (Eckes et al., 2000; Desmouliere et al., 2005), and tumorigenesis (Beacham and Cukierman, 2005; Gaggioli et al., 2007; Yamada and Cukierman, 2007).

Cells interacting with 3D collagen matrices exhibit distinct patterns of cell signaling (Cukierman et al., 2002; Wozniak et al., 2003; Beningo et al., 2004; Rhee et al., 2007) and increased plasticity of cell migration (Sahai and Marshall, 2003; Shreiber et al., 2003; Friedl, 2004; Even-Ram and Yamada, 2005; Zaman et al., 2006; Wolf et al., 2007). We have been studying human foreskin fibroblast migration in nested collagen matrices (Grinnell et al., 2006). To prepare nested matrices, contracted collagen matrices known as dermal equivalents (Bell et al., 1979) are polymerized within cell-free outer matrices. Cells can migrate from the dermal equivalents into the outer matrices. Platelet-derived growth factor (PDGF) is unique among growth factors in its capacity to promote human fibroblast migration in nested collagen matrices, whereas sphingosine-1-phosphate acts as an inhibitor of migration (Jiang et al., 2008).

Time-lapse microscopic observations of fibroblasts migrating in nested collagen matrices showed that in addition to cell migration, collagen fibril flow occurred in the outer matrix toward the dermal equivalent boundary. Features of this flow suggested that it depends on the same cell motile machinery normally used by cells for migration. Collagen fibril flow was capable of producing large-scale tissue translocation as shown by closure of a ~1-mm gap between paired dermal equivalents in floating, nested collagen matrices. Our findings demonstrate that tractional force exerted by fibroblasts in collagen matrices can couple differentially to cell migration or matrix translocation.

MATERIALS AND METHODS

Materials

Type I collagen (3 mg/ml, Vitrogen) was purchased from Cohesion (Palo Alto, CA). DMEM, CO2-independent DMEM, and 0.25% trypsin/EDTA solution were purchased from Invitrogen (Gaithersburg, MD). Fetal bovine serum (FBS) was purchased from Gemini (Woodland, CA). Rho kinase inhibitor Y-27632 was obtained from Calbiochem-Novabiochem (La Jolla, CA). Blebbistatin was obtained from Toronto Research Chemicals (Toronto, Canada). PDGF BB iso type was obtained from Upstate Biotechnology (Lake Alfred, CA). PDGF BB iso type was obtained from Upstate Biotechnology (Lake Alfred, CA).
matrix and then covered with the remaining 140/200-

Briefly, dermal equivalents were formed by contraction for 6–48 h of

performed as described previously (Figure 1A; Grinnell

incubated in DMEM (or CO2-independent DMEM for time-lapse studies) con-

matrices containing 1.5 mg/ml collagen (Figure 1B). Nested matrices were

coated beads.

was not required, and no differences were observed compared with albumin-

software (Molecular Devices, Menlo Park, CA). The cell migration index was

collected with a Nikon Elipse 400 fluorescence microscope (Melville, NY) and

2006). Images of PI-stained cells used for quantification of cell migration were

phalloidin and PI was carried out as previously described (Grinnell

Preparation of samples for actin staining with Alexa Fluor 594–conjugated

from the bottom of the culture surface using a spatula. For time-lapse imag-

translocation, the image field was calibrated with a micrometer slide, and

single 2D video. For quantitative analysis of cell migration and collagen

from a video (Sup_1.mov) in which individual cells can be

seen migrating from the dermal equivalent into the outer

interface between dermal equivalent (darker area) and outer matrix (lighter area). As described previously, a wave of fibroblast migration across the interface typically began after several hours (Grinnell et al., 2006). Migrating cells all were localized within the outer collagen matrix. No direct interaction occurred between migrating cells and the underlying culture dish on which nested collagen matrices were restrained.

RESULTS

Fibroblast Migration in Nested Collagen Matrices

Figure 1A diagrams the nested collagen matrix model of cell migration in which dermal equivalents—fibroblast-contracted floating collagen matrices—are embedded in cell-free, outer collagen matrices. Figure 1B presents the typical appearance viewed from above of a nested collagen matrix with the dermal equivalent (dense inner region) surrounded by an outer matrix (more translucent region). Figure 1C shows time-lapse phase-contrast images focused at the inter-

Collagen Translocation in Nested Collagen Matrices

Figure 2A presents representative, phase-contrast images from a video (Sup_1.mov) in which individual cells can be seen migrating from the dermal equivalent into the outer matrix. Migrating cells typically had leading dendritic extensions that were branched. Some of these extensions increased in size and became stabilized during migration; others regressed. In addition to cell migration, collagen translocation occurred in the outer matrix toward the dermal equivalent interface. Collagen movement can be appreciated by noting the position of matrix deformations (Figure 2A, asterisks). In the video, collagen translocation has the appearance of collagen fibril flow.

Collagen translocation in the outer matrix toward the dermal equivalent occurred earlier than fibroblast migration. Figure 2B shows this relationship quantitatively by measuring cell migration and collagen translocation during 1-h intervals averaged from three separate videos. The data demonstrate that the velocity of collagen translocation peaked before cell migration began. Once cells begin to emerge from the inner matrix, the velocity of collagen translocation declined.
The decrease in velocity of collagen translocation might have been a feature of the experimental system independent of the time when cell migration began. To test this possibility, studies were carried out using nested collagen matrices prepared with dermal equivalents that had been contracted for 48 h. With 48-h dermal equivalents, a longer lag phase precedes cell migration compared with the 6-h dermal equivalents used for the experiments described in Figures 1 and 2. Figure 3A shows representative images from the beginning and end of a time-lapse video (Sup_2.mov) in which nested collagen matrices were prepared with 48-h contracted dermal equivalents containing 3-µm polystyrene beads (diluted 1:1000) in the outer matrix to help visualize collagen matrix flow. Figure 3B presents quantification of the results. If nested collagen matrices were floating in

**Figure 2.** Cell migration and collagen translocation in nested matrices. (A) Representative images of the times indicated from a time-lapse microscopic video (Sup_1.mov) of nested collagen matrices (6-h dermal equivalent) showing individual cells migrating from the dermal equivalent into the outer matrix. Collagen translocation can be appreciated by noting the position of matrix deformations (labeled by asterisks) that move toward the interface with the dermal equivalent. Bar, 25 µm. (B) Comparison of collagen translocation and cell migration. Average ± SD of three separate movies showing changing velocities (displacement measured during 1-h intervals) of collagen translocation (circles) and cell migration (squares). For each movie, velocities were determined by measuring 3 points at the interface, 10 points in the outer matrix, and the tail ends of six cells. When cells first appeared in the outer matrix, the distance measured was from the cells’ tail ends to the interface. Data were corrected for movements of the interface.

**Figure 3.** Collagen translocation in nested matrices. (A) Representative images from the beginning and end of a time-lapse video (Sup_2.mov) in which nested collagen matrices were prepared with 48-h contracted dermal equivalents containing 3-µm polystyrene beads (diluted 1:1000) in the outer matrix to help visualize collagen matrix flow. (B) Collagen movement in the outer matrix toward the dermal equivalent was quantified by measuring the displacements (average ± SD) of 10 different beads (arbitrarily selected) at 1-h intervals. Data were corrected for movements of the interface. Bar, 130 µm.

**Collagen Flow Depends on Actin, Myosin, and Rho Kinase**

Studies were carried out to learn more about the cellular mechanisms responsible for collagen flow. Movement of collagen was actin-dependent because addition of cytochalasin D (5 µM) after 7 h completely inhibited further cell or collagen translocation (Sup_3.mov.). We also compared the effects of pharmacologic inhibitors of myosin II and Rho kinase on collagen translocation under conditions previously shown to inhibit fibroblast migration in nested collagen matrices (Grinnell et al., 2006). Figure 4 shows that blebbistatin, which blocks myosin II activity, completely prevented both cell migration and collagen translocation. Y27632, which blocks Rho kinase activity, partially inhibited cell migration and collagen translocation. These findings were consistent with the idea that cells use the same motile machinery for collagen translocation as for cell migration.

**Cell Migration in Restrained Versus Floating Nested Collagen Matrices**

The observations described in Figures 1–4 all were made using nested collagen matrices restrained on culture dish surfaces. We and others have shown that fibroblast physiology and morphology differ markedly when fibroblasts interact with collagen matrices floating in culture medium versus restrained on culture dishes (Grinnell, 2003). Therefore, we compared cell migration with nested collagen matrices that were restrained on culture dishes or floating in culture medium. Figure 5A shows fluorescence images of samples stained by PI, and Figure 5B presents quantification of the results. If nested collagen matrices were floating in
medium, then the cell migration index was reduced markedly compared with restrained conditions.

Additional experiments were carried out with restrained and floating nested collagen matrices that contained paired dermal equivalents embedded ~1 mm apart in outer matrices. Figure 6A shows the experimental design and presents immunofluorescence images taken at the edge and gap regions of the paired matrices after culture for 24 h. Figure 6B shows quantification of the findings. With restrained, paired nested matrices, cell migration occurred to a similar extent around the edges of the dermal equivalents and in the gap between. With floating, paired nested matrices, migration was limited almost entirely to the gap region in between. Taken together, the findings in Figures 5 and 6 suggested that fibroblast migration in collagen matrices depended on the ability of the matrix to resist tractional force exerted by the cells. Resistance could be provided by restraint of nested matrices on culture surfaces or by the opposition between paired dermal equivalents if the nested matrices were floating in culture medium.

**Collagen Matrix Reorganization and Tissue Translocation**

Even though there was little cell migration in floating compared with restrained nested matrices, extensive collagen reorganization occurred under floating conditions. Figure 7A shows fluorescence images of nested matrices in which the outer matrix contained 6-µm fluorescent beads. Imme-

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**Figure 4.** Myosin II and Rho kinase dependence of cell migration and collagen translocation. (A) Nested collagen matrices were prepared with 22-h contracted dermal equivalents and incubated 20 h in medium containing PDGF plus 10 µM Rho kinase inhibitor Y-27632 or 20 µM myosin II inhibitor blebbistatin as indicated. Subsequently cells were fixed and stained with PI. Bar, 100 µm. (B) Cell migration index values shown are the averages ± SD of duplicate samples from two separate experiments. (C) Average ± SD of two separate movies showing changing velocities (displacement measured during 1-h intervals) of collagen translocation. For each movie, velocities were determined by measuring 10 points in the outer matrix and 3 points at the interface. Data were corrected for movements of the interface.

**Figure 5.** Cell migration in floating versus restrained nested matrices. (A) Nested collagen matrices (24-h dermal equivalent) were incubated 24 h, after which cells were stained with PI. Bar, 100 µm. (B) Cell migration index values shown are the averages ± SD of duplicate samples from three separate experiments.

**Figure 6.** Cell migration in floating and restrained, paired nested collagen matrices. (A) Paired nested collagen matrices prepared with 19-h dermal equivalents were incubated 35 h in medium containing 50 ng/ml PDGF either restrained on culture dishes or floating in medium as indicated. At the end of the incubations, cells were fixed and stained with PI to visualize cell nuclei. (B) Cell migration index values shown are the averages ± SD of duplicate samples from two separate experiments and seven microscopic fields per sample. Bar, 60 µm.
Immediately after nested matrices were prepared (0 h), beads were distributed uniformly throughout the outer matrix. After 24 h, cell migration was observed in restrained nested matrices, but little bead accumulation at the dermal equivalent interface could be detected. Conversely, in the case of floating nested matrices, little cell migration was observed, but beads accumulated along the interface between the dermal equivalent and outer matrix. Particle counts made using NIH Image J software (http://rsb.info.nih.gov/ij/) on several fields from duplicate matrices showed that compared with newly prepared nested collagen matrices, bead density across the interface increased slightly more than twofold in 24-h restrained matrices and more than 10-fold in 24-h floating nested matrices.

We also tested the consequences of collagen reorganization using floating, paired nested matrices. Figure 8, A and B, shows representative images from the times indicated in time-lapse microscopic videos of experiments with 19-h dermal equivalents (Sup_4.mov) and 48-h dermal equivalents (Sup_5.mov). Figure 8A shows that cell migration occurred in the gap between the 19-h dermal equivalents, tension lines formed, and some gap closure took place. Figure 8B shows that with paired nested collagen matrices containing 48-h dermal equivalents, little cell migration occurred but gap closure was complete. These finding indicated that collagen translocation in the nested matrices could result in large-scale tissue movement.

For human fibroblasts interacting with collagen matrices, PDGF acts as a promigratory growth factor, whereas LPA and FBS are procontractile and show little stimulation of migration (Grinnell et al., 2006). Additional experiments were carried out to compare the effects of these agonists on gap closure with paired, nested collagen matrices. Figure 8C shows that neither LPA- nor FBS-stimulated gap closure. Also, no gap closure occurred if paired, nested matrices were restrained.

**DISCUSSION**

Fibroblasts and other cells can remodel collagen matrices mechanically. Remodeling has been demonstrated at a global level by measuring collagen matrix contraction (Cukierman et al., 2002; Grinnell, 2003). Remodeling also has been studied by observing local changes in collagen organization in response to cell motile activity (Roy et al., 1997; Tamariz and Grinnell, 2002) and cell migration (Gaggioli et al., 2007; Wolf et al., 2007). In addition, if fibroblast explants are embedded in collagen matrices, then collagen fibrils in between the explants become aligned into linear tracks (Stopak and
Harris, 1982; Sawhney and Howard, 2002). In the current article, we describe another aspect of mechanically remodeling of collagen matrices: collagen fibril flow and large-scale tissue translocation. In the case of floating nested matrices, collagen fibril flow was sufficient to permit closure of a 1-mm gap between paired dermal equivalents.

Under some circumstances, collagen flow has been reported to occur within collagen matrices independent from tissue cells (Newman et al., 1985). The collagen flow we detected in the current studies required fibroblasts and appeared to utilize the same cell motile machinery involved in cell migration. Interfering with actin or inhibiting myosin II or Rho kinase inhibited cell migration and collagen translocation. In addition, the growth factor specificity of collagen flow was similar to cell migration. Gap closure in floating, paired nested matrices took place in the presence of PDGF but not LPA or FBS. Previously, we showed that PDGF but not LPA or FBS stimulates human fibroblast migration in nested collagen matrices (Grinnell et al., 2006). Serum frequently has been used as an agonist to study cell migration, and PDGF is believed to be the major promigratory factor for fibroblasts in serum (Li et al., 2004; Gao et al., 2005). However, serum also contains the lipid growth factor sphingosine-1-phosphate (Eichholtz et al., 1993; Yatomi et al., 1997). SIP1 recently was shown to be a potent inhibitor of human fibroblast migration in collagen matrices and to reduce the promigratory activity of serum (Jiang et al., 2008).

Evidence regarding the regulatory role that Rho kinase plays in cell migration is complex. Blocking Rho kinase activity can inhibit or stimulate cell migration depending on cell type and experimental conditions (Riento and Ridley, 2003; Tosukawara et al., 2004). Besides for collagen fibril flow and cell migration, Rho kinase activity also is required for PDGF-stimulated fibroblast-collagen matrix contraction (Rhee and Grinnell, 2006). Human fibroblasts in collagen lack detectable Rho activation upon PDGF stimulation (Grinnell et al., 2003). Therefore, basal rather than agonist-stimulated Rho kinase probably is required for cell migration and contraction, perhaps by maintaining basal levels of myosin light chain phosphorylation (Abe et al., 2003; Knock et al., 2008).

Collagen fibril flow not only appeared to depend on the same cell motile machinery as cell migration, but also tended to occur reciprocally with migration. That is, collagen translocation was greatest before cells began to migrate in the outer matrices. Using more contracted dermal equivalents to extend the period before cell migration begins increased the period of collagen flow. Why collagen flow eventually decreased under the latter conditions even in the absence of cell migration remains to be determined. In the case of floating nested matrices, we observed decreased cell migration and increased collagen flow. Collagen flow in paired nested collagen matrices resulted in dermal equivalent gap closure if the matrices were floating.

Why there is a lag phase before cell migration begins, and why the length of the lag phase increases along with the time of dermal equivalent contraction are questions yet to be resolved. One possibility is that during the lag phase, changes occur in the cells or matrix required for fibroblasts to move from across the interface between the dermal equivalent whose collagen density can be as high as ∼25 mg/ml collagen (Ahlfors and Billiar, 2007) into the outer collagen matrix composed of 1.5 mg/ml collagen. On collagen-coated planar surfaces, cells tend not to move from stiffer to softer materials (Lo et al., 2000). Whatever the final explanation, we believe that the studies with restrained compared to floating nested matrices indicate an important role for tension. Fibroblasts in floating collagen matrices have fewer stress fibers and focal adhesions than cells in restrained matrices (Grinnell, 2003). Based on the finding that cellular stress fibers and focal adhesions are indicators of cell tension (Singer et al., 1984; Burridge et al., 1988; Balaban et al., 2001; Galbraith et al., 2002), fibroblasts are less able to develop tension in floating compared with restrained matrices. Given that tension plays a positive role in cell migration on planar surfaces (Tucker et al., 1985; Kolega, 1986; Belousov et al., 2000; Lo et al., 2000; Wang et al., 2001; Raaber et al., 2007), the decreased ability of cells in floating nested collagen matrices to develop tension (unless the dermal equivalents are paired) provides a reasonable explanation for the lack of migration.

Figure 9 offers what we believe to be an attractive hypothesis to account for our overall observations. If the collagen matrix can resist cellular tractional force, then the cells can move. If the matrix cannot resist cellular traction force, then the matrix moves. See text for additional details.
tissue (cf. Peacock, 1984), a cell-dependent version of what surgeons call “mechanical creep” (Johnson et al., 1993; Wilhelmi et al., 1998).

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