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Effects of PDGF-BB on Rat Dermal Fibroblast Behavior in Mechanically Stressed and Unstressed Collagen and Fibrin Gels

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The dose–response effects of platelet-derived growth factor BB (PDGF-BB) on rat dermal fibroblast (RDF) behavior in mechanically stressed and unstressed type I collagen and fibrin were investigated using quantitative assays developed in our laboratory. In chemotaxis experiments, RDFs responded optimally ($P < 0.05$) to a gradient of 10 ng/ml PDGF-BB in both collagen and fibrin. In separate experiments, the migration of RDFs and the traction exerted by RDFs in the presence of PDGF-BB (0, 0.1, 1, 10, or 100 ng/ml) were assessed simultaneously in the presence or absence of stress. RDF migration increased significantly ($P < 0.05$) at doses of 10 and 100 ng/ml PDGF-BB in collagen and fibrin in the presence and absence of stress. In contrast, the effects of PDGF-BB on RDF traction depended on the gel type and stress state. PDGF-BB decreased fibroblast traction in stressed collagen, but increased traction in unstressed collagen ($P < 0.05$). No statistical conclusion could be inferred for stressed fibrin, but increasing PDGF-BB decreased traction in unstressed fibrin ($P < 0.05$). These results demonstrate the complex response of fibroblasts to environmental cues and suggest that mechanical resistance to compaction may be a crucial element in dictating fibroblast behavior. © 2001 Academic Press

Key Words: **cell traction; cell migration; chemotaxis; fibroblast; PDGF-BB; tissue equivalent; fibrin; collagen; wound healing.**

INTRODUCTION

Dramatic differences have been observed between cells entrapped within the network of fibrils comprising type I collagen and fibrin gels as compared to cells cultured on glass and plastic, including morphology [1, 2], proliferation rates [3, 4], and protein synthesis [5–7]. Cells interact with these fibrillar proteins by exerting traction through bound integrins [8–11]. This traction can lead to compaction of the network and/or migration of the cells through the network [12–15]. These mechanical phenomena play important roles in tissue morphogenesis, tissue engineering, and wound healing. For instance, in dermal wound healing, fibroblasts are stimulated to migrate up a chemotactic gradient of soluble factors released by inflammatory cells and platelets into a provisional matrix composed primarily of fibrin and fibronectin. These fibroblasts proliferate and secrete collagen and other extracellular matrix molecules to form granulation tissue. The cells often contract this granulation tissue while continuing to secrete collagen [16]. Ultimately, the cells die through apoptosis and leave a dense, collagenous, acellular scar as a reparative patch [17]. The progression of fibroblast behavior is dictated in part by cues from the wound healing environment, such as soluble growth factors, integrin binding to network proteins, and mechanical stress associated with any wound contraction [16].

Previous studies have elucidated the roles of some of these factors on fibroblast behavior. For instance, Mochitate *et al.* demonstrated that the presence of mechanical stress in attached collagen gels induces prominent alignment of cytoskeletal actin bundles, whereas the absence of stress in free floating collagen gels results in highly disorganized actin [18]. (The mechanical stress more precisely develops in the network of entangled fibrils comprising the gel, although we will refer to these simply as "stress" and "network.") Also, numerous studies have investigated the effects of soluble peptide growth factors on cell behavior. Platelet-derived growth factor BB (PDGF-BB) is one such peptide growth factor that is released from platelet granules and inflammatory cells at the wound site. PDGF-BB has been shown to be mitogenic [19, 20], chemotactic [20–23], and chemokinetic [21] for fibroblasts *in vitro*, and it increases compaction of unstressed collagen [24–26]. PDGF-BB has also been shown *in vitro* to differentially affect expression of integrins involved in binding to collagen and fibronectin [27]. Conversely, the ability of another growth factor, EGF, to modulate fibroblast migration has been

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shown to depend on the concentration of extracellular matrix molecules and, hence, integrin binding [28, 29]. Further, studies with TGF-β have demonstrated that the effects of a growth factor on protein expression and gel compaction can be modulated by the presence of stress in the network [30].

Despite these observations, the roles that environmental cues such as matrix protein type, mechanical stress, and growth factors play in modulating specific behavior remain largely unknown, primarily because of the lack of quantitative assays to study these phenomena. We have developed two experimental assays which enable us to quantitatively assess chemotaxis, migration, and traction in stressed and unstressed type I collagen and fibrin gels [13, 31, 32]. In this investigation of rat dermal fibroblast (RDF) behavior, we report on the dose–response relationships between PDGF-BB and chemotaxis in unstressed collagen and fibrin and between PDGF-BB and RDF migration and traction in stressed and unstressed collagen and fibrin. We conclude that the effects of PDGF-BB on RDF chemotaxis and migration are similar in collagen and fibrin, but that the effects on traction depend upon the combination of network stress and network type. The results indicated that the effects of PDGF-BB on cell traction are dictated in part by the mechanical resistance of the collagen or fibrin to compaction.

METHODS

Materials

Trypsin (25 mg/ml in 0.9% saline), ethylenediaminetetraacetic acid (EDTA), $CaCl_2$, NaOH, bovine fibrinogen, bovine thrombin, and agarose were purchased from Sigma Chemical Company (St. Louis, MO). Vitrogen 100 bovine type I collagen was from Cohesion Technologies, Inc. (Palo Alto, CA). Tissue culture medium, penicillin/streptomycin (pen-strep), fungizone, Hepes buffer, phosphate-buffered saline (PBS), and L-glutamine were from Gibco Laboratories (Grand Island, NY). Fetal bovine serum (FBS) was from HyClone Laboratories (Logan, UT). Recombinant human PDGF-BB was purchased from R&D Systems (Minneapolis, MN).

Cell Culture

RDFs were obtained using a primary explant technique [33] and kept in liquid nitrogen after slow freezing. Cell lines were initiated for culture by thawing an aliquot of cells and centrifuging at 1000 rpm for 10 minutes at 4°C. The pellet of cells was resuspended in Dulbecco's modified Eagle's medium (DMEM) supplemented with pen-strep (1% v/v) and L-glutamine (1% v/v). Cells were plated on 60 × 15-mm petri dishes using 5 ml of DMEM with 10% FBS, 1% pen-strep, and 1% L-glutamine and kept in a humidified CO_2 incubator at 37°C. Trypsin/EDTA was used to passage the cells once a week at a 1:4 dilution. Cells were harvested with 0.5% trypsin/EDTA and washed twice with Medium 199 (M199) with Hanks' salts supplemented with 10% FBS, 1% pen-strep, and 1% L-glutamine. Cells were passed or harvested at 60–90% confluence. All experiments were conducted before the ninth passage, at which point a new culture was initiated from frozen cells.

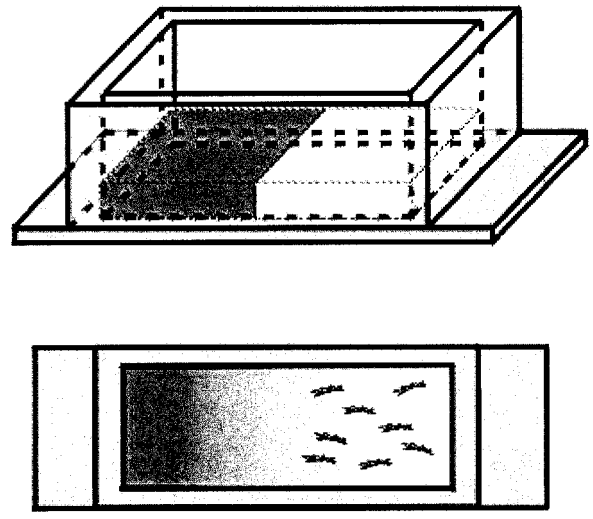

FIG. 1. Schematic of the chamber used for chemotaxis assays. A rectangular chamber was separated into two halves by a polycarbonate divider. One half was filled with gel solution containing a defined concentration of PDGF-BB. After gelation, the divider was removed and the other half was filled with a gel solution containing dispersed cells. PDGF-BB was able to diffuse freely into the conjoined gel.

Gel Preparation

Collagen gels. Collagen gels were prepared as described previously [34]. Briefly, Vitrogen 100 was neutralized with 0.1 N NaOH and diluted to 2.0 mg/ml collagen using cell culture medium (M199 and 10× MEM supplemented with 6% FBS) to obtain physiological pH and ionic strength. Cells were then dispersed in the collagen-forming solution at the desired concentration. The collagen solution was placed in a 37°C incubator to permit gelation.

Fibrin gels. Fibrin gels of 3.3 mg/ml concentration were made as described previously [32]. Briefly, fibrinogen powder was dissolved in a Hepes-buffered saline solution at a concentration of 30 mg/ml. The resulting solution was passed through a 0.20-μm filter and aliquoted. Thrombin powder was dissolved in 10 ml of Hepes-buffered saline to yield a solution of 25 units/ml. The thrombin solution was then filtered through a 0.20-μm syringe filter and aliquoted. Aliquots were stored at −80°C. Upon use, the fibrinogen aliquots were diluted to 5 mg/ml using M199 supplemented with 12% FBS to yield a fibrinogen solution containing 10% FBS. The thrombin aliquots were diluted to 2.5 units/ml in M199 containing 15 mM Ca^{2+}. To create fibrin gels, four parts fibrinogen solution were mixed with one part thrombin solution and one part cell suspension (or medium without cells for the creation of acellular gels) and placed in an incubator at 37°C to facilitate gelation.

For those gels, either collagen or fibrin, which were employed in cell tracking studies, 20-μm polystyrene beads (Polysciences, Warrington, PA) were dispersed along with the cells as a tracer to allow gel convection to be subtracted from the measured cell displacement [13].

Chemotaxis Assay

Chemotaxis studies were performed as reported previously [31, 32]. Briefly, the chemotaxis chambers consisted of a polycarbonate box mounted on top of a microscope slide using silicone-based vacuum grease (Dow Corning, Midland, MI) as shown in Fig. 1. A removable polycarbonate divider was used to separate the chamber into two halves. A collagen or fibrin solution containing PDGF-BB was allowed to gel in one-half of the chamber. The polycarbonate

divider was then removed and a collagen or fibrin gel containing dispersed RDFs at a concentration of 10^4 cells/ml was created in the other half. PDGF-BB was free to diffuse from the cell-free to the cell-containing half of the conjoined gel. A microscope slide was sealed to the top of the chamber with silicone grease to maintain sterility during the incubation period. Cells were incubated in the chamber for approximately 36 h. Cell movement was tracked with 3-D automated single-cell tracking (described below) during the incubation period, or cell orientation measurements were taken at the end of the 36-h incubation period.

RDF alignment was measured in both the presence and the absence of PDGF-BB gradients. For gradient experiments, PDGF-BB was added to the cell-free half of the chamber but not the cell-containing half. Experiments were also performed in the absence of PDGF-BB and in the presence of a uniform concentration of PDGF-BB (PDGF-BB added equally to both halves of the chamber). For the case of uniform concentration, the PDGF-BB concentration used was the equilibrium concentration in the gradient studies (one-half the loading in the gradient studies since half the gel was initially unloaded). After a 36-h incubation period, pictures of the gel were taken with a 4× objective with a Photometrics SenSys CCD camera (Tucson, AZ) mounted on an Olympus IX-70 inverted light microscope (Melville, NY). Images were taken at 75-μm intervals throughout the depth of the gel. These individual images were combined using Inovision ISee image analysis software (Raleigh, NC) into a single *x-y* projection composite image of the gel. Using this composite image, cell alignment was measured as the angle, α, between the long axis of the cell and the direction of the gradient. An alignment parameter was calculated as $\phi = \sin^2 \alpha$, and an average alignment parameter, Φ, was determined for each experiment by averaging ϕ over approximately 200 cells in each chemotaxis chamber. A randomly oriented population of cells has a Φ value of 0.5, whereas Φ values below 0.5 indicate an average cell alignment along the direction of the gradient [32]. Cell tracking studies in selected experiments using the same methods described below were used to ascertain if alignment was associated with orientation up or down the gradient. (The general lack of morphological polarity complicates orientation assessment from still images, although alignment is easily determined because the cells are highly elongated.)

Simultaneous Cell Traction and Migration Assay

Studies in stressed gels were performed as described previously [13]. A schematic of an assay chamber is shown in Fig. 2. Briefly, the assay consisted of a cell-seeded cylindrical collagen or fibrin gel that was prevented from compacting axially. Two cylindrical stainless-steel posts provided the axial constraint with porous polyethylene platens attached to the ends. During stressed gel preparation, the collagen- or fibrin-forming solution penetrated the porous polyethylene resulting in a firm bond between the posts and the collagen or fibrin upon gelation. Chambers were autoclaved and polycarbonate sheaths were soaked in FBS for approximately 30 min at 37°C to block potential binding sites on their surfaces. The chambers were then assembled and a fibrinogen/thrombin/cell or collagen/cell suspension was pipetted inside the polycarbonate sheath. Since fibrin has a higher network modulus than collagen [35], a higher cell concentration was used in the fibrin gels in order to generate a similar level of macroscopic compaction to that observed in the collagen gels. The chambers were placed inside an incubator at 37°C to facilitate gelation. Following gelation, a glass coverslip was attached to the bottom of the chamber with vacuum grease, and M199 with 10% FBS and known concentrations of PDGF-BB (0–100 ng/ml) were placed in the chamber well. The polycarbonate sheath was then withdrawn and a coverslip was attached to the top of the chamber with vacuum grease. Chambers were then placed in the incubator for approximately 2 h before being transferred to the automated time-lapse video microscopy system.

As a comparison to the mechanically constrained cylindrical gels, free-floating cylindrical gels were also created. Because compaction

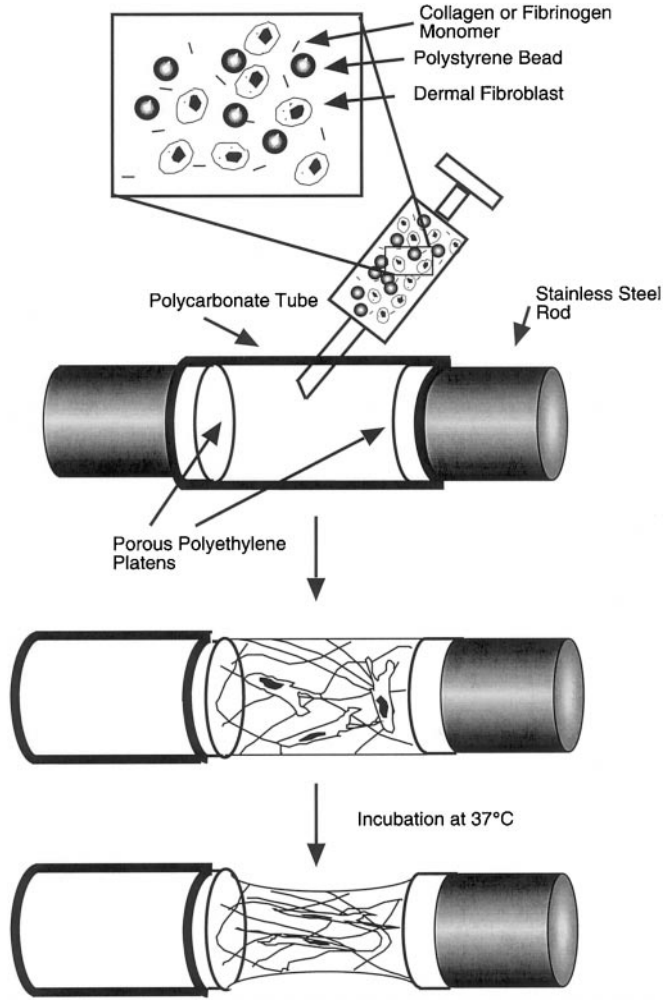

FIG. 2. Schematic of the chamber used to prepare gel samples for stressed traction/migration assays. A polycarbonate sheath was filled with collagen or fibrinogen solution containing RDFs and polystyrene beads. The sheath was supported at either end by fixed posts. After gelation, the chamber was filled with culture medium, and the sheath was slid over one of the posts, exposing the formed gel to the culture medium.

is unconstrained for free-floating gels, no mechanical stress is generated in the gel as cells exert traction, and the gel compacts uniformly in all directions. Assay chambers for unstressed gel preparation were identical to stressed gel chambers with one exception. Smooth-ended polycarbonate posts were used in place of the porous polyethylene-capped stainless-steel posts. The polycarbonate posts were soaked in FBS for approximately 30 min at 37°C to block potential binding sites on their surfaces. As a result, the fibrin and collagen gels did not adhere to the posts. Unstressed gels compact more rapidly than their stressed counterparts at the same cell concentrations [13]. Therefore, lower cell concentrations were used in unstressed gel experiments to maintain similar levels of macroscopic gel compaction as in the stressed cases. The final modification of the traction assay protocol for the fabrication of unstressed gels involved the addition of 0.5% (w/v) agarose to the tissue culture medium to increase the viscosity of the medium. This served to limit bulk convective movement of the free-floating gels that would otherwise hinder high-magnification time-lapse measurements of cell migra-

tion. A preliminary set of experiments using stressed gels ($n = 3$) demonstrated that the presence of agarose did not affect gel compaction or cell migration (unpublished data).

High-Magnification 3-D Single-Cell Tracking

High-magnification cell tracks of fibroblasts within collagen or fibrin gels in either chemotaxis or traction assay chambers were generated as described elsewhere [32, 36]. A Zeiss Axiovert 135 inverted light microscope (Thornwood, NY) with an automated stage and KP-M2U CCD Hitachi camera (Woodbury, NY) was coupled to a Kontron IBAS image analysis workstation (Thornwood, NY) running a macro for time-lapse cell tracking. Briefly, using the 40× objective, several cells were selected randomly from within the gel and their position was recorded. Each cell was associated with a neighboring (within 400 μm) polystyrene bead. The time-lapse loop was then initiated. At the specified time interval, the cell-tracking program would move the microscope stage to the last identified cell position and execute an autofocus routine to adjust the *z*-position of the stage. Then, a series of image analysis routines were used to identify the centroid of the cell. The cell positions were updated and recorded. The total time-lapse period was typically 24 h, and a typical time-lapse interval was 45 min. At the end of the time-lapse experiment, the cell displacement information was corrected for gel convection effects by subtracting the displacement of the polystyrene bead co-localized with the given cell.

Cell Migration Analysis

From a set of cell tracks like those in Fig. 4b, the mean-squared displacement, $\langle d^2(t)\rangle$, of the cells was computed. It was assumed in the simultaneous cell traction and migration studies that RDF migration could be modeled as a persistent random walk which for displacement in three dimensions is related to the interval time, t according to:

$$\langle d^2(t)\rangle = 6\mu[t - P(1 - e^{-t/P})], \quad (1)$$

where μ is the random migration coefficient of the cells and P is the directional persistence time [37, 38]. At times much greater than the persistence time of the cell (~2–3 h for RDFs), as was the case of our experiments, the slope of the mean-squared displacement plot is proportional to the random migration coefficient. The random migration coefficient was computed via a generalized least-squares regression (GLSR) of the asymptotic mean-squared displacement versus time data and provided a single parameter for making quantitative comparisons regarding the migratory response of RDFs under different experimental conditions [38].

Low-Magnification Time-Lapse of Compacting Gels

Low magnification time-lapse video microscopy of macroscopically compacting gels was conducted concurrently with high-magnification cell tracking. The motorized nosepiece of a Zeiss Axiovert 135 inverted microscope allowed the objective to be automatically changed from 40× to 2.5×. A mosaic image of the gel was taken at the beginning of each time-lapse interval. Midplane diameter measurements of the gel at each time point were taken from these images and used to determine midplane compaction of the gel. Quantitative comparisons between conditions were based on the final midplane compaction.

Statistics

To account for changes in baseline cell behavior, chemotaxis, migration, and traction, data were analyzed with a two-way, mixed-model ANOVA, with PDGF-BB concentration as a fixed effect and the day of the experiment as a random effect. Pairwise comparisons between individual concentrations were performed with Fisher's least significant difference (LSD) post hoc analysis. Significance levels were set at $P < 0.05$. Due to differences in cell concentration, statistical comparisons were only performed within each of the four possible combinations of conditions (collagen or fibrin, stressed or unstressed).

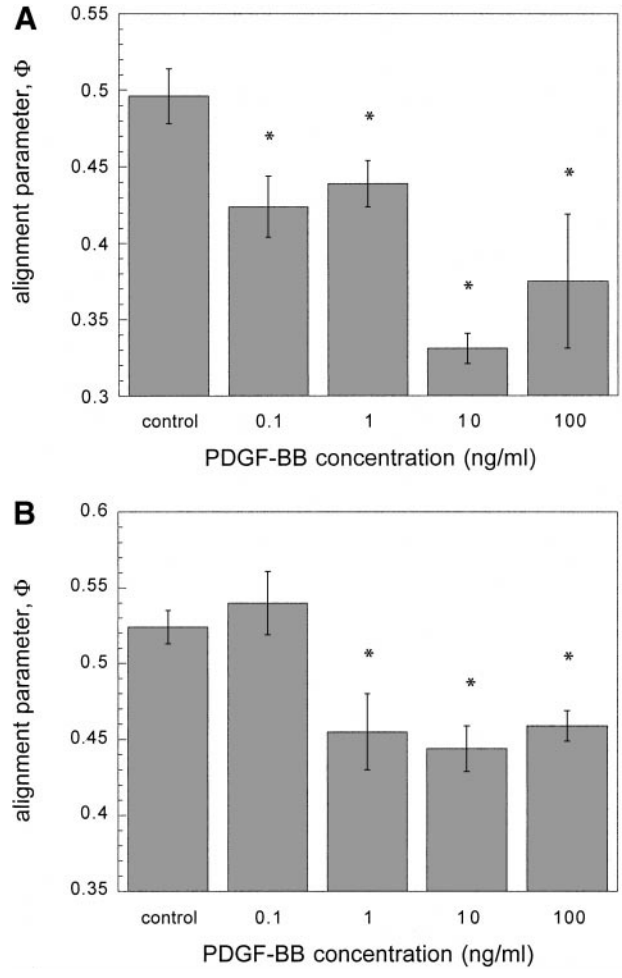

FIG. 3. Mean cell alignment parameter ± SD for RDFs in the presence of 0–100 ng/ml PDGF-BB gradient loadings. (A) In collagen, RDFs demonstrated a significant difference in alignment at loadings of 0.1 ng/ml PDGF-BB and greater. (B) In fibrin, RDFs demonstrated a significant change in alignment at 1 ng/ml PDGF-BB and greater. In both collagen and fibrin control cases (no PDGF-BB added), the cell orientation parameters were not significantly different from a random orientation value of 0.5.

RESULTS

Chemotaxis

Cell alignment. RDF alignment was measured in response to PDGF-BB loadings ranging from 0.1 to 100 ng/ml. Figure 3 shows average cell alignment, Φ, after a 36-h incubation as a function of initial PDGF-BB concentration in the cell-free half of the chamber for both collagen and fibrin. RDFs were aligned with the

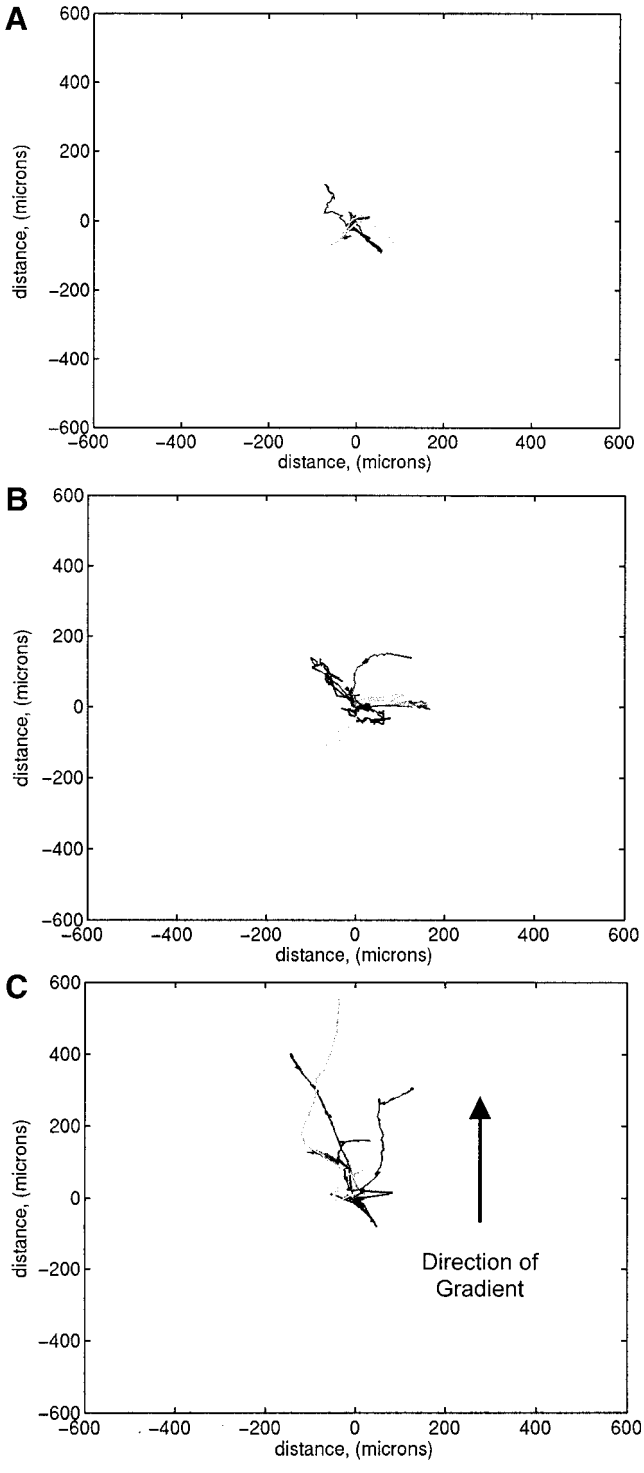

FIG. 4. Two-dimensional projections of three-dimensional cells tracks during 36-h incubation in fibrin under different chemotactic conditions. Each line represents the migration of a different cell. (A) In the absence of PDGF-BB, cells only migrated a few cell lengths in random directions. (B) In the presence of a uniform concentration of 5 ng/ml PDGF-BB, cells migrated approximately twice as far as in the absence of PDGF-BB, but still in a random direction. (C) In the presence of a gradient loading of 10 ng/ml PDGF-BB, cell migration was stimulated and directed up the PDGF-BB concentration gradient, indicating a positive chemotactic response.

PDGF-BB gradient at PDGF-BB loadings of 0.1 ng/ml and greater in collagen and 1.0 ng/ml and greater in fibrin with maximum alignment occurring at 10 ng/ml in both cases. However, the alignment was markedly weaker in fibrin compared to collagen. In the absence of a PDGF-BB gradient (no PDGF-BB present), Φ was not statistically different from the random orientation value of 0.5. Assuming that cell alignment was dominated by cells oriented toward higher concentrations of PDGF-BB, these data suggest that RDFs exhibit a positive chemotactic response at concentrations of 0.1 ng/ml and above in collagen and 1.0 ng/ml and above in fibrin.

Cell tracking. Two-dimensional projections of 3-D cell tracks acquired during a 36-h incubation in fibrin are shown in Fig. 4. Note that initial cell positions have been translated to the origin. In the absence of PDGF-BB, RDFs did not move more than a few cell lengths (Fig. 4A). Cell tracks for RDFs exposed to a uniform concentration of 5 ng/ml PDGF-BB are presented in Fig. 4B. The cells migrated more than twice as far as those not exposed to PDGF, yet their direction of migration was random.

In Fig. 4C, tracks of cells exposed to a PDGF-BB gradient loading of 10 ng/ml are shown. As with the cells exposed to uniform PDGF concentrations, these cells migrated farther than in the absence of PDGF-BB. However, the migration of the cells was clearly biased up the PDGF gradient, confirming a positive chemotactic response. RDFs also responded chemotactically to a 100 ng/ml gradient, but the distance of migration was about half that seen with 10 ng/ml and the migration was less biased in the direction of the gradient. The same behavior was seen in collagen (data not shown). These cell tracks confirm the results of the cell alignment studies and indicate that RDFs exhibit a maximal chemotactic response to PDGF-BB at 10 ng/ml in collagen and fibrin gels.

Traction and Migration

Through the use of an automated light microscope with software-controlled magnification, we obtained gel compaction data at low magnification while simultaneously performing cell tracking at high magnification. PDGF-BB concentrations studied ranged from 0 to 100 ng/ml. Cell track data in collagen containing a uniform concentration of PDGF-BB are shown in Fig. 5 along with the GLSR fits of Eq. (1). The cell migration coefficients computed from the data presented in Fig. 5 are listed in Table 1. Typical compaction plots for

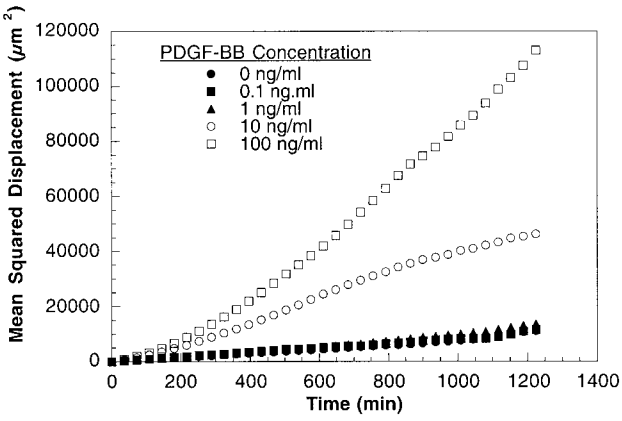

FIG. 5. Representative mean squared displacement plots for cells in stressed collagen exposed to different concentrations of PDGF-BB. Random migration coefficients were determined from these curves by modeling cell migration as a persistent random walk. In this experiment, as observed generally for all conditions examined (stressed and unstressed, collagen and fibrin), the addition of PDGF-BB stimulated RDF migration.

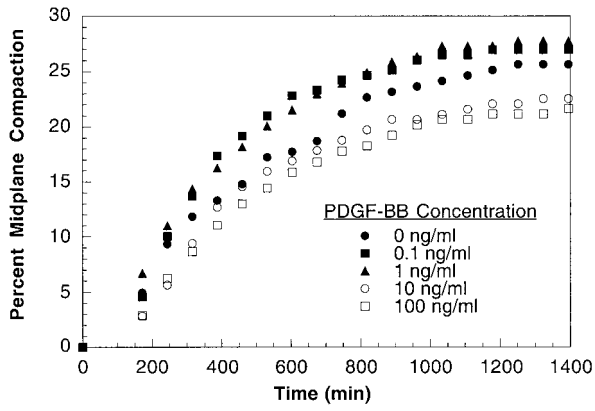

FIG. 6. Representative midplane compaction over time for stressed collagen. Midplane compaction of gels was calculated by measuring the midplane diameter from mosaic images taken approximately every 45 min for 24 h. In this experiment, as observed generally for stressed collagen, compaction decreased with increased PDGF-BB concentration.

stressed collagen assays using various PDGF-BB concentrations are shown in Fig. 6. Gel compaction over time is generally a function of the number of cells, the traction exerted per cell, and the protein type and concentration of the gel (due to differences in mechanical properties and possibly biochemical properties that might alter the cell traction). Therefore, if the gel type, network concentration, and cell number are held constant across experiments, the compaction of the gel provides a measure of the per cell traction.

As demonstrated in Figs. 7 and 8, the average effect of a particular concentration of PDGF-BB on cell traction or migration was associated frequently with a large standard error. The experiments and statistical analysis were designed to identify and account for error associated with random differences in cell behavior and gel properties among experiments (i.e., on different days). First, the effects of varied PDGF-BB concentration were assessed only within an experimental day. Replicate experiments were then performed on subsequent days and the data were analyzed with a mixed-model, two-way ANOVA (experiment day was treated as a random effect and PDGF-BB concentration as a fixed effect). We observed statistically significant differences in both cell migration and gel compaction as a function of not only PDGF-BB concentration, but also experiment day in most cases, indicating that the day-to-day variation in baseline cell behavior and/or gel properties were a large contributor to the observed error. Table 2 summarizes the two-way ANOVA results for migration and compaction. The variation as a function of PDGF-BB concentration is discussed below. The day-to-day variation in cell behavior is attributed mainly to variation in cell viability, cell passage number, cell confluence, and possibly variations in gel properties. Although specific steps were taken to minimize these effects by using cells of uniformly high viability, similar passage number, and similar confluence at harvest time, the statistical analysis reveals that these effects remained significant. Nevertheless, significant trends were gleaned from the data by accounting for the day-to-day variation in the statistical analyses.

TABLE 1

Random Migration Coefficient, μ, Calculated from the Mean-Squared Displacement (MSD) Data Shown in Fig. 5 for RDFs in Collagen

PDGF-BB concentration (ng/ml)	Random migration coefficient, μ (μm^2/min)
0.0	1.4 ± 0.2
0.1	1.5 ± 0.2
1.0	1.5 ± 0.2
10.0	3.8 ± 0.4
100.0	4.9 ± 0.8

Collagen. Cell concentrations used in both the stressed and the unstressed collagen traction assays are shown in Table 3. Average final midplane compaction measurements for stressed and unstressed collagen at various PDGF-BB concentrations are shown in Fig. 7A; cell migration coefficient data are presented in Fig. 7B. For stressed collagen, increasing PDGF-BB concentration resulted in decreased compaction (which implies decreased cell traction) and increased cell migration. The critical PDGF-BB concentration was between 1 and 10 ng/ml for both compaction and migration. Very similar results were observed with respect to migration in the unstressed collagen. However, the

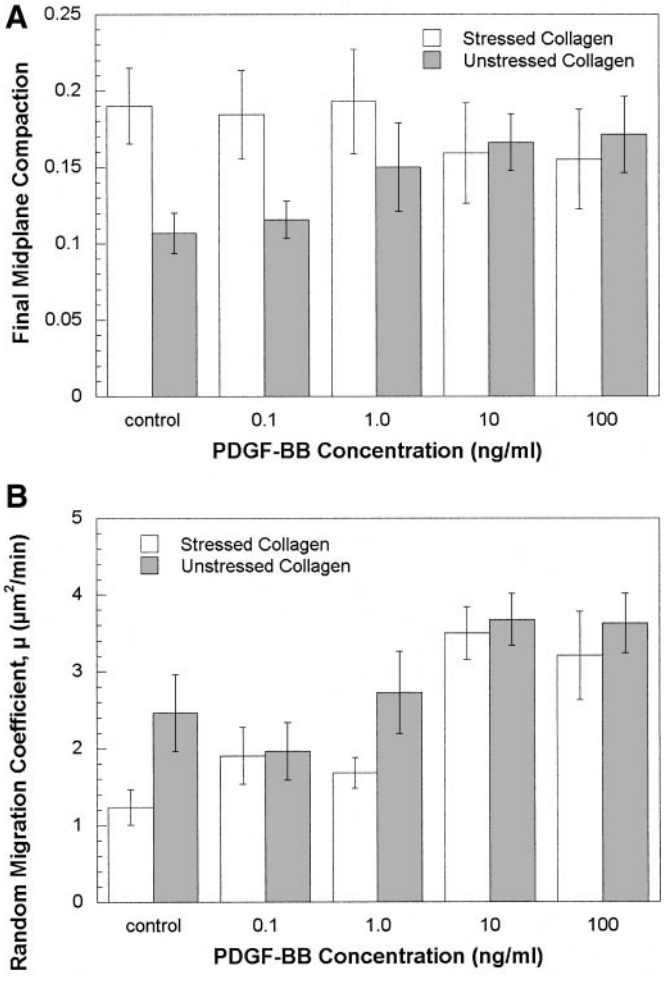

FIG. 7. Effects of PDGF-BB on cell behavior in collagen gel. (A) Mean ± SD compaction for stressed and unstressed collagen. Traction decreased at higher concentrations of PDGF-BB in stressed collagen, but increased in unstressed collagen. (B) Mean ± SD migration coefficient for stressed and unstressed collagen. Migration increased at higher concentrations of PDGF-BB in both stressed and unstressed collagen. Error bars in these plots include error from the effects of different basal responses of cells. These "experimental day effects" are accounted for in the statistical analysis of the data, which is presented in detail in Table 2.

results of the compaction study in the unstressed gels were the opposite of those observed in the stressed ones. As PDGF-BB concentration increased in the unstressed gels, gel compaction also increased. (Due to differences in both the anisotropy of compaction and the cell concentration between stressed and unstressed gels, these data do not indicate whether per cell traction was greater in stressed or unstressed collagen at a given PDGF-BB concentration. Quantitative comparisons of cell traction based on midplane gel compaction can only be made within a specific type of experiment, for example, stressed collagen.)

Fibrin. Cell concentrations used in both the stressed and the unstressed fibrin traction assays are shown in Table 3. Average final midplane compaction and cell migration data for RDFs in stressed and unstressed fibrin are presented in Figs. 8A and 8B, respectively. As for collagen, RDF migration increased with increasing PDGF-BB concentration in both the stressed and unstressed fibrin. While a statistical analysis is not possible due to the differing cell numbers, gel types, and network concentrations, it is interesting to note that RDF migration in fibrin was lower than in the corresponding collagen assays. In contrast to the results for collagen, there was no statistically significant difference in compaction as a function of PDGF-BB in stressed gels. In the unstressed gels, gel

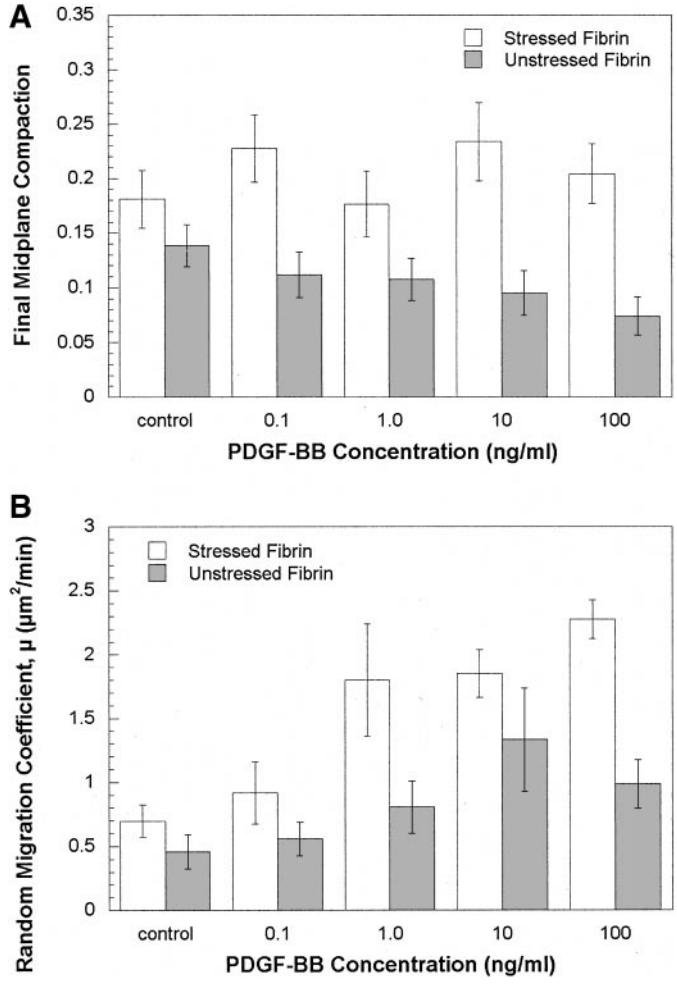

FIG. 8. Effects of PDGF-BB on cell behavior in fibrin gel. (A) Mean ± SD compaction for stressed and unstressed fibrin. No trend was observed for traction in stressed fibrin. Traction decreased at higher concentrations of PDGF-BB in unstressed fibrin. (B) Mean ± SD migration coefficient for stressed and unstressed fibrin. Migration increased at higher concentrations of PDGF-BB in both stressed and unstressed fibrin. Error bars in these plots include error from the effects of different basal responses of cells. These "day effects" are accounted for in the statistical analysis of the data, which is presented in detail in Table 2.

TABLE 2

Results of the Mixed-Model, Two-Way ANOVA for the Individual Assays

Gel type	Stress state	Cell behavior	Effect	*P* value
Collagen	Stressed	Migration	[PDGF-BB]	0.003*
			Day	0.002*
		Traction	[PDGF-BB]	0.004*
			Day	0.0001*
Collagen	Unstressed	Migration	[PDGF-BB]	0.0001*
			Day	0.066
		Traction	[PDGF-BB]	0.044*
			Day	0.0001*
Fibrin	Stressed	Migration	[PDGF-BB]	0.002*
			Day	0.424
		Traction	[PDGF-BB]	0.461
			Day	0.090
Fibrin	Unstressed	Migration	[PDGF-BB]	0.029*
			Day	0.016*
		Traction	[PDGF-BB]	0.001*
			Day	0.0001*

Note. Significance levels were set at $P < 0.05$.

compaction decreased with increasing PDGF-BB concentration, which was the opposite of the response seen in the analogous, unstressed collagen assay. Therefore, it appears that cell traction was unaffected by PDGF-BB in stressed fibrin but decreased in response to PDGF-BB in unstressed fibrin. (Again, no direct comparison of the magnitude of cell traction in stressed and unstressed fibrin can be made solely upon this compaction data, nor are direct comparisons of compaction data between fibrin and collagen assays valid.)

DISCUSSION

A comprehensive characterization of the effects of PDGF-BB on RDF chemotaxis, migration, and traction has been presented. Similar chemotactic behavior to PDGF-BB was observed for fibroblasts in both type I collagen and fibrin. PDGF-BB increased the migration of the fibroblasts in both type I collagen and fibrin in both the presence and the absence of mechanical stress in the fibrillar network. Cell traction, inferred from the percentage of compaction of the gels, was affected significantly by PDGF-BB for all but the stressed fibrin; however, the nature of the effects was dependent on the gel protein type and the presence of stress. The results of the study are summarized qualitatively in Table 4.

The chemotactic response of RDFs to PDGF-BB substantiates previous studies using Boyden chambers or similar assays [20–23, 39]. However, our chemotaxis model differs from these in that: (1) cells are migrating in a three-dimensional protein network similar to the *in vivo* condition; (2) the strength of chemotaxis is described quantitatively by the cell alignment parameter Φ; (3) the gradients are shallower than in Boyden chambers and, arguably, more like those *in vivo;* and (4) cell motion can be tracked directly in three dimensions. The first item is especially important in light of reports that describe PDGF-BB-dependent alterations of integrin expression that could differentially affect adhesion to ECM molecules [27]. Interestingly, RDFs did not respond to PDGF-BB until a concentration of 1–10 ng/ml which is similar to other studies, suggesting that PDGF-BB receptor expression may not be affected dramatically by the gel type or dimensionality of the substratum.

The dose–response relationship between PDGF-BB and fibroblast migration was similar to the chemotactic response. We previously compared fibroblast migration in a stressed type I collagen gel in the presence and absence of a single dose (50 ng/ml) of PDGF-BB and fibroblast migration in stressed versus unstressed collagen independently—migration increased under both of these conditions [13]. However, the current study represents the first quantitative assessment of the interdependence of fibroblast migration on PDGF-BB concentration, the presence of network stress, and the gel type. PDGF-BB increased fibroblast migration in all conditions relative to the control condition (no PDGF-BB added) for each case. Generally, based on post hoc pairwise comparisons, the critical dose was between 1 and 10 ng/ml PDGF-BB, which was the same optimum concentration range for chemotaxis. Thus, the response of RDFs to PDGF-BB may be optimized to produce rapid as well as oriented migration.

While it is difficult to draw direct comparisons among the different conditions because of different cell concentrations used to equalize compaction rates between fibrin and collagen, it is interesting that we observed an increase in the control migration in unstressed versus stressed collagen. This corroborates our previous results [13]. However, no stress-dependent trends were seen in fibrin controls. Moreover, fibroblasts were generally more motile in collagen than fibrin. The observed differences between the migration of cells in fibrin and collagen are likely due to either intrinsic cell mechanisms triggered by different inte-

TABLE 3

Cell Concentrations Used in Simultaneous Traction and Migration Assays

Gel type	Stress state	Initial cell concentration (cells/ml)
Collagen	Stressed	15,000
	Unstressed	13,000
Fibrin	Stressed	60,000
	Unstressed	20,000

TABLE 4

Summary of Effects of Increasing PDGF-BB Concentration on Cell Behavior

	Unstressed			Stressed		
	Chemotactic alignment	Migration	Traction	Chemotactic alignment	Migration	Traction
Collagen	+	+	+	n/a	+	−
Fibrin	+	+	−	n/a	+	No effect

Note. +, increase, and −, decrease with increasing PDGF-BB concentration.

grin peptide binding sequences or, more generally, a response to substrate compliance. These factors also may play a role in the development of traction by fibroblasts.

To that end, the novelty of our methods is that they allow simultaneous quantification of migration and compaction in free-floating, mechanically unstressed gels or attached, mechanically stressed gels. Generally, given the same cell concentration, network compliance, and mechanical constraint to compaction, a greater amount of compaction implies a greater amount of per cell traction [13]. Our ability to quantify traction and migration simultaneously enabled us to examine potential relationships between these two phenomena. There has been considerable debate concerning the "driving force" behind gel compaction. Some studies suggest that cell migration drives compaction [40, 41]; however, these studies are based primarily on inferring a relationship between cell morphology and increased gel compaction or direct force measurements, rather than a direct measurement of migration. Others suggest that stationary, contractile, "myofibroblasts"—fibroblasts expressing a smooth-muscle-cell-like phenotype—are responsible for gel compaction [42]. Specifically, these fibroblasts demonstrate well-defined smooth muscle actin filaments in their cytoskeleton which are believed to mediate cell traction [43]. Myofibroblasts have been implicated in wound contraction and other fibrocontractive disorders [44].

The results presented herein suggest a relatively complex relationship between cell migration and gel compaction. It is likely that a number of elements are critical in orchestrating the varied responses, but we hypothesize that the mechanical resistance in the gel network is the factor that determines whether stimulation of migration with PDGF-BB results in an increase or decrease in cell traction. In particular, we hypothesize that the traction generated by the stimulated migratory phenotype is greater than that of the low-traction phenotype dominant in low-resistance environments, but less than that of the high-traction phenotype found in high-resistance environments.

Our hypothesis is based primarily on two observations of RDF behavior:

In the absence of stimulatory factors such as PDGF-BB, RDFs in a gel are in a "default" traction phenotype that depends on the resistance perceived by the cell as it interacts with the substrate. If a cell perceives sufficient resistance, it enters a high-traction, myofibroblastic phenotype; if not, it maintains a low-traction phenotype that is less efficient at gel compaction. It has been well established that fibroblasts in mechanically stressed gels have a well-defined network of smooth muscle actin consistent with a myofibroblastic phenotype, whereas fibroblasts in stress-free gels generally have loosely organized actin networks [18, 30, 45]. Recent work from our laboratory demonstrated that, consistent with the phenotypic observations described above, fibroblasts exert more traction in mechanically stressed collagen than unstressed collagen, even though cells in both conditions are able to compact the gel [13].

Stimulating RDFs with PDGF-BB overrides the default traction phenotype in favor of an intermediate traction, migratory one. This observation is supported by the data presented in this paper where PDGF-BB stimulated migration regardless of the gel type and state of mechanical stress. Unstimulated cells may still migrate. In fact, we have observed that unstimulated fibroblasts migrate more in a mechanically stress-free gel than in a stressed one. However, the migration of cells in the default traction phenotype is suboptimal and can be significantly enhanced with the addition of PDGF-BB. The traction associated with this phenotype is the traction required for the cell to migrate through the network.

With these two principles in place, the effect of PDGF-BB in each combination of gel type and mechanical stress can be examined with two questions in mind. First, what is the default phenotype of unstimulated cells? Second, assuming that the traction exerted by cells as they migrate can also compact a gel, how does the traction generated by PDGF-BB-stimulated, migrating cells compare relative to the traction generated by unstimulated cells? It is again important to note that we are not comparing the absolute traction or amount of compaction among the various conditions (Table 2), but only how stimulating cells with

PDGF-BB alters the amount of compaction relative to the appropriate control.

First, in unstressed collagen, the mechanical resistance is low, which induces a low-traction default phenotype—cells are relatively inefficient at generating traction compared to the same cells in a stressed gel [13]. Stimulation with PDGF-BB induces a migratory phenotype, and the current study and previous ones have observed that stimulation of fibroblasts with PDGF-BB increases gel compaction in unstressed collagen [24–26]. It is therefore relatively straightforward to conclude that the traction exerted by stimulated, migrating cells is greater than that of unstimulated, low-traction cells in this low-mechanical resistance environment.

Next, in stressed collagen, the default phenotype is a high-traction one due to the high mechanical resistance in the gel network resulting from the mechanical constraint. These cells exert more traction and are therefore more efficient at compacting the gel compared to cells in unstressed collagen [13]. Stimulation of migration with PDGF-BB decreased the compaction of stressed collagen compared to the compaction from the default, high-traction cells. We propose that inducing a change away from the high-traction, myofibroblastic phenotype, such as inducing a migratory phenotype with PDGF-BB, will result in less traction exerted on the network. Previous work from our laboratory demonstrated that in stressed collagen, where the default phenotype is expected to be high traction, other conditions under which fibroblasts shifted to a migratory phenotype (inhibition of cell adhesion with an antibody against the β1 integrin subunit) also resulted in decreased compaction [13, 35].

Based on the results of unstressed collagen, it might be expected that fibroblasts in unstressed fibrin are in a default low traction state and that stimulating migration with PDGF-BB will lead to an increase in gel compaction. However, while a mechanically stressed gel resulting from constrained compaction is one condition under which cells experience a significant resistance to compaction, another is an intrinsically stiff network. As a cell exerts traction on a stiff network, it will again perceive significant resistance at its attachment sites even if the network itself is not mechanically stressed by a physical constraint. At the gel protein concentrations used in this investigation (3.3 mg/ml fibrin and 2 mg/ml type I collagen), the fibrin network is approximately three times as stiff as the collagen network in their initial states [35]. Therefore, we hypothesize that the increased stiffness of the fibrin gel induces a default high-traction phenotype even though the gel is unstressed. Stimulation with PDGF-BB drives the cell away from this high-traction phenotype toward a migrating one of intermediate traction just as in stressed collagen.

The final case to consider is stressed fibrin. Clearly, as this is both a stiff and mechanically constrained gel, the high-traction default state of fibroblasts would be expected, and a decrease in gel compaction is expected after stimulating cells to migrate just as in stressed collagen and unstressed fibrin. As noted earlier, we observed no difference in migration between stressed and unstressed fibrin in the absence of PDGF-BB which is consistent with the view that the default cell phenotype is the same under both stress conditions in fibrin in contrast to collagen. However, no clear relationship was observed between PDGF-BB and compaction in stressed fibrin. Because of the compounding effects of the stiff fibrin network and the constrained compaction, a much higher traction is needed to compact the gel to the same degree as unstressed fibrin or either stressed or unstressed collagen. This is reflected in the significantly higher cell concentration used in the stressed fibrin gel compared to all other cases as shown in Table 3. The higher cell concentration may introduce secondary effects such as greater cell–cell contacts or increased paracrine signaling which could complicate the results and obscure the relationship that otherwise would be observed at lower cell concentrations. These secondary effects may account for the lack of a trend between PDGF-BB concentration and compaction in stressed fibrin. Unfortunately, there is no simple way to probe the effect of PDGF-BB on the compaction of the stiff, stressed fibrin network without using relatively high cell concentrations. Also, we have focused on the biomechanical determinants of cell phenotype in relation to traction. It is quite possible that the results observed in fibrin are different from those in collagen predominantly because of biochemical differences in the gel protein type and not biomechanical ones. We are currently exploring ways to modulate the stiffness of the network without affecting its biochemical properties so that we can more effectively study the effect of network stiffness on default cell phenotype.

Our hypothesis assumes that the effects of PDGF-BB on gel compaction are mediated through an alteration in cell traction by stimulation of fibroblast migration. It has been proposed that the stimulation of cell motility by growth factors involves the coordinated regulation of substrate adhesion and intracellular contractile forces [46, 47]. Clearly, both of these components will affect the resistance perceived by the cell, as well as the net traction imposed on the gel. PDGF-BB has been shown to modulate the types of integrins expressed by fibroblasts on various matrices [27] as well as to induce dynamic reorganization of cytoskeletal forms of actin involved in cell motility [48–51]. Moreover, for many tissue cells including vascular smooth muscle cells and fibroblasts, PDGF-BB has been shown to inhibit smooth muscle actin expression [52–55]. These findings are consistent with our hypothesis that PDGF-BB

induces cells of intermediate traction compared to unstimulated cells in high and low mechanical resistance environments. The downregulation of smooth muscle actin by PDGF-BB would produce a decrease in traction compared to unstimulated cells in high mechanical resistance environments, where a well-defined network of smooth muscle actin has been observed. However, the upregulation of cytoskeletal elements involved in active cell migration by PDGF-BB would produce more traction than unstimulated cells in a low mechanical resistance environment, where smooth muscle actin expression is limited [18, 30, 45]. PDGF-BB induced alterations in integrin expression may also give rise to differential integrin signaling, and feedback from integrin signaling may also modulate PDGF receptor expression or the cell's response to PDGF-BB binding. The presence of such feedback is quite likely, but its effects are not expressly accounted for by our biomechanical hypothesis.

It is apparent that the behavior of fibroblasts in tissue equivalents is not easily characterized by one variable, such as network stress or growth factor concentration. Rather, cells appear programmed to exhibit distinct behavior based on the specific combination of environmental cues. A deeper understanding of these cues can lead to an increased ability to control cell behavior. As such, we may be able to dictate the degree of wound contraction or improve the properties of artificial tissues fabricated from cell-compacted collagen and fibrin [56]. Future work will focus on identifying the sensitivity to and synergy among some of these cues, such as network stiffness and adhesivity.

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