A Novel Implantable Collagen Gel Assay for Fibroblast Traction and Proliferation during Wound Healing

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INTRODUCTION

Wound healing comprises a complex interaction of cellular, biomechanical, and biochemical phenomena. Fibroblasts play a central role in wound healing by producing the extracellular matrix (ECM) molecules that ultimately replace the damaged tissue, by first migrating into the wound site and proliferating (fibroplasia) and then depositing new matrix and restructuring the tissue [1]. As part of the process of closure, wounds may undergo contraction, giving rise to scarring and loss of function [2, 3]. While the exact mechanism of wound contraction is still in dispute, it is now widely accepted that fibroblast traction provides the driving force [4].

Due to the complexity of the wound healing response, we have developed a number of in vitro assays that allow the quantitative measurement of selected cell behaviors important in wound healing. They all employ 3-D collagen or fibrin gels to provide a physiologically relevant experimental ECM. These include assays for chemotaxis [5, 6], contact guidance [7], traction [8, 9], and migration [8, 10]. We have recently used some of these assays to conduct a systematic investigation of the role of platelet-derived growth factor (PDGF) BB in modulating fibroblast traction and migration [11].

While in vitro collagen and fibrin gel assays provide a more physiologically relevant means of studying the role of growth factors such as PDGF BB in the wound healing response compared to monolayer cell culture on glass or plastic, they fail to capture several critical features of the wound. First, the assays are oversimplified in that they include interactions among a single combination of cell, ECM, and growth factor types. This shortcoming could be rectified by coculturing cells, using ECM mixtures, or adding multiple growth fac-
tors, but such efforts would increase experimental complexity without necessarily providing a commensurate increase in biofidelity nor yielding greater insight into the wound healing process. Second, our assays are designed to provide fairly static conditions in contrast to the highly dynamic wound environment. Most notable is the continuous change in the composition of the soluble wound environment. For example, PDGF AA is present at elevated levels immediately after wounding that rapidly decline over the following 48 h [12]. While this change in composition could be mimicked by sequentially exposing in vitro gel assays to wound fluids from different postwound days, the result would be a discontinuous change in soluble wound mediator concentrations with increased potential for loss of growth factor activity under culture conditions.

A number of different in vivo models have been developed to study wound healing. These assays offer the advantage of an actual wound environment in contrast to the idealized one provided by in vitro systems. However, the ability to make quantitative measurements of cellular behavior is limited. Commonly, a model animal is wounded and some element of the repair process such as the rate of wound closure, the wound breaking strength, or the histology is observed [13–15]. Other models involve the implantation of some device in the wound site to act as a repository for wound fluid and as a structure onto which granulation tissue can form. These include the polyvinyl alcohol sponge [16, 17], the polyurethane sponge [18], the cellulose sponge [21], expanded polytetrafluoroethylene tubing [22, 23], the stainless steel wire mesh cylinder [24], and their variants. The implantable models are primarily useful for collecting wound fluid, monitoring inflammation, and studying fibroplasia and matrix deposition [25]. Only the nonimplant wound models provide any insight into wound contraction and then only if the wound boundary is fixed or has been marked, since contraction and matrix deposition can occur simultaneously. Further, these models provide no way to estimate cell traction from observed wound contraction since neither the cell density nor the tissue mechanical properties are precisely known. Finally, none of them permit fibroblasts to be exposed to soluble wound factors while interacting with a defined matrix that can be withdrawn and characterized at a later time.

An assay that combined the ability to quantify cell behavior and the soluble wound environment would be ideal. Herein, we present an assay in which a fibroblast-seeded collagen gel is enclosed in a semipermeable tube that is inserted into a sponge wound model and, thereby, exposed to the dynamic soluble wound environment. This assay precludes the ability to continuously monitor individual cells, but it does provide a means for estimating cell traction and measuring cell proliferation for cells exposed to the actual soluble wound environment.

**MATERIALS AND METHODS**

**Materials.** Trypsin (25 mg/ml in 0.9% saline), ethylenediaminetetraacetic acid (EDTA), CaCl2, NaOH, agarse, anti-cy-smooth muscle actin monoclonal antibody, and TRITC-conjugated phaloidin were purchased from Sigma Chemical Co. (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). Cored cylindrical cellulose vicose (CV) sponges were from Celldomed (Turku, Finland). Nalgene PVC tubing was from Fisher Scientific (Pittsburgh, PA). Teflon thread was from McMaster-Carr (Chicago, IL). Sodium pentobarbitol was from Abbott Laboratories (Chicago, IL). Polyvinylidene difluoride (PVDF) dialysis membranes were from Spectrum Laboratories (Rancho Dominguez, CA). Propidium iodide and goat anti-mouse Alexa 488 antibody were from Molecular Probes (Eugene, OR).

**Cell culture.** Rat dermal fibroblasts (RDFs) were harvested from adult male Fisher rats using a primary explant technique [26] 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 min 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 out on 60 × 15-mm petri dishes using 6 ml of DMEM with 10% FBS, 1% pen-strep, and 1% L-glutamine and kept in a humidified CO2 incubator at 37°C. Trypsin/EDTA was used to pass the cells once a week at a 1:4 dilution. Cells were harvested with 0.5% trypsin/EDTA and washed twice with complete medium (Medium 199 containing Hanks’ salts (M199) 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 10th passage, at which point a new culture was initiated from frozen cells.

**Sponge wound model preparation.** Cored CV sponges were placed in double-distilled water, allowed to swell, and then cut in half to yield two cylindrical cored sponges of approximately 2 cm in length. One-quarter-inch i.d. rubber tubing was cut into approximately 3-mm-thick cylindrical sections. Nalgene tubing of 3/16-in. i.d. × 1/4-in. 0.d. dimension was cut into approximately 3.5-cm lengths. The rubber tubing was placed over one end of the Nalgene tubing and two sutures were used to attach one set of tubing to each end of a CV sponge as shown in Fig. 1. Teflon thread of 1/16 in. diameter was cut to 8.5-cm length and placed inside the spongötube assembly. The whole assembly was then placed in double-distilled water and auto
Collagen gel preparation. Collagen gels were enclosed within 4-mm flat width hollow-fiber PVDF dialysis membranes with a 106-Da molecular weight cut-off so as to permit unhindered passage of all but the very largest soluble factors present in the wound environment yet prevent inflammatory and tissue cells from invading or transplanted fibroblasts from leaving the gel. The flat membranes were expanded to a cylindrical geometry by flushing them with water. Membranes were rinsed in double-distilled water to remove traces of sodium azide from the storage solution and then wetted in 70% ethanol for approximately 45 min. Following ethanol wetting, care was taken to prevent the membranes from drying. The membranes were transferred to double-distilled water bath, autoclaved, and kept in sterile water at 4°C until ready for use. A 0.5% agarose solution was prepared by dissolving low-melting agarose in 25 mM Hepes-buffered saline, the solution was then autoclaved. Specially designed heating blocks containing 30.1-cm-diameter by 2.5-cm-deep holes were autoclaved, and 2 ml of sterile agarose was pipetted into each hole. The agarose-filled heating block was then transferred to a 4°C environment for at least an hour to induce agarose gelation. About 2 h before the experiment, the heating block was placed in an incubator to elevate the agarose temperature to 37°C. Just prior to the start of the experiment, the heating block was transferred to a dry bath set at 37°C.

Collagen solution at a concentration of 2.0 mg/ml was prepared from Vitrogen 100 type I collagen. Twenty microliters of 1 M Hepes block was transferred to a dry bath set at 37°C. Just prior to the start of the experiment, the heating environment for at least an hour to 37°C was then transferred to a 4°C saline solution. Tubes were kept in the saline solution until ready for implantation approximately an hour later. Following anesthetization of the rat, two small incisions were made near the ends of the Nalgene tubing. The thermoplastic adhesive was removed from the ends of the Nalgene tubes. The PVDF membrane enclosing the collagen gel was then attached to the Teflon thread inside the spongotubing assembly with a ligating clip (Ethicon, Somerville, NJ), and the thread was pulled out the other side of the tubing until the gel was centered in the sponge. Excess Teflon thread was removed, the ends of the Nalgene tubing were resealed with thermoplastic adhesive, and the small incisions were closed with surgical staples.

Collagen gel removal and measurement. Gels were removed at either 2 or 4 days following their implantation in the CV sponge. The animal was sacrificed by CO2 asphyxiation and the incision was made on its dorsal side. The sponge assembly was removed and the PVDF-enclosed gel was taken from the sponge and placed in 20 mM Hepes-buffered saline. Microscissors were used to cut open the PVDF membrane, and the gel was carefully removed from the membrane and maintained in saline.

An Olympus IX-70 inverted light microscope (Melville, NY) with automated stage and Photometrics cooled CCD camera (Tucson, AZ) was used to obtain a transmitted light mosaic image of the gels such as the ones shown in Fig. 4. Images of several gels were also taken immediately following gel formation in order to establish the initial size. Inovision iSee image analysis software (Raleigh, NC) was used to measure the gel diameter by averaging gel diameter measurements taken at three points along the length of the gel. The percentage compaction was then estimated by dividing the average compacted gel diameter by the average initial diameter. Statistical comparisons of compaction data was performed with ANOVA. Significance levels were set at P < 0.05.
Cell counts. Cells were counted using an adaptation of a previously developed technique [27]. Briefly, each gel was placed in 50 μl of 0.5 mg/ml collagenase and incubated at 37°C for 1 h. The cells were fixed by adding 65 μl of 4% paraformaldehyde, and 2 μl of propidium iodide was added to stain the cell nuclei. The cells were spun down in a well of a 96-well tissue culture plate, and an image of the well was taken under fluorescence illumination. Image analysis software was used to count the visible nuclei in the image. Statistical comparisons of cell counts were performed with ANOVA. Significance levels were set at \( P < 0.05 \).

Traction estimation. Our lab has previously developed a theory for cell-matrix mechanical interactions such as occur in the fibroblast-seeded collagen gel employed in this assay [28], and we have subsequently extended this theory to account for matrix fiber alignment that can occur during compaction [29]. However, because the gels employed in this assay are free-floating within the PVDF membrane (i.e., unconstrained), their compaction is uniform and no alignment should develop [29]. It is, therefore, sufficient to use the isotropic version of the theory for the data presented herein (see also Barcas et al. [30]). The details of the biphasic theory are presented in the Appendix. Briefly, five conservation equations (for cells and for mass and momentum of the fiber network and interstitial solution phases) are combined with two constitutive equations (network viscoelasticity and cell spreading) to relate the macroscopic compaction of the gel to the cell concentration and the average per-cell traction represented by the cell traction parameter, \( r_c \). Material properties for collagen gel have already been obtained by our lab as have estimates of cell spreading (i.e., time dependence of \( r_c \)) and the cell migration coefficient [8, 31]. A summary of the parameter values used is presented in Table 1.

Using initial cell number and cell count data from the harvested gels, we can estimate a first-order rate constant for cell proliferation for each experimental condition by fitting the cell proliferation data to the integrated form of the equation

\[
\frac{dn}{dt} = k_0 n,
\]

where \( n \) is the cell number, \( t \) is time, and \( k_0 \) is the cell proliferation rate constant. Given the initial cell concentration, we can then use the biphasic theory to estimate \( r_c \) from the gel compaction data while accounting for the different cell proliferation rates under our various experimental conditions. The system of equations was solved using an FEM model combined with the COOPT optimization routine as described elsewhere [32, 33].

Actin staining. Recovered gels were fixed for 60 min in 4% paraformaldehyde and then rinsed with PBS containing 0.3% Triton (PBST). Gels were then blocked for 60 min with 10% normal goat serum. Anti-α-smooth muscle actin monoclonal antibody was diluted 1:100, and the gels were incubated in this antibody solution overnight. The next day, gels were rinsed with PBST before being incubated for 60 min with goat anti-mouse Alexa 488 secondary antibody at a 1:100 dilution. TRITC-conjugated phalloidin was also added at a 1:100 dilution to stain for F-actin [34]. Gels were then rinsed with PBST and mounted on a slide with DAPI mounting medium (Vector Laboratories, Burlingame, CA).

Mechanical testing. An Instron (Canton, MA) planar biaxial tester configured in uniaxial mode with resolution on the order of 0.1 mN was used to determine the ultimate tensile strength of the recovered gels as described elsewhere [27]. Briefly, the gels were gripped with spring-loaded tissue grips and stretched at a constant strain rate until failure. Reported stress values were based upon estimated gel cross-sectional area prior to stretching.

RESULTS

Two postwounding time frames were investigated: the 1- to 5-day period and the 7- to 11-day period. In both cases, the time of wounding (CV sponge implantation) was designated day 0. For the 1- to 5-day period, PVDF tubes containing gels were inserted into the implanted sponge on the 1st day after wounding and harvested on the 3rd and 5th days. For the 7- to 11-day time frame, gels were inserted on the 7th day and harvested on the 9th and 11th days. Paired gels were maintained in culture in complete medium for comparison.

Gel compaction. Average gel diameter over time is presented in Fig. 5 for the two time frames. Three gels were used to estimate average gel diameter at each time point. In the day 1–5 wounds, the implanted gels compacted more rapidly by day 3 and by day 5 than gels cultured over the same time (ANOVA, \( P < 0.02 \)). However, the gels implanted in day 7–11 wounds compacted at about the same rate as gels cultured over the same time. The amount of compaction in day 1–5 wounds was also significantly greater than compaction in day 7–11 wounds (ANOVA, \( P < 0.02 \)). Compaction did not statistically differ between in vitro controls for day 1–5 and day 7–11 experiments.

Cell number. Cell counts for the various gels are presented in Fig. 6. Cells from three gels were counted to estimate average cell number at each time point. In day 1–5 wounds, cell number increased approximately 30% in the implanted gels while there was essentially no change in cell number in gels cultured during the same time. This increase was not statistically significant (ANOVA, \( P = 0.11 \)). In day 7–11 wounds, there
was a significant increase ($P < 0.001$) in cell number of about 160% over the 4-day implantation period while no significant change in cell number occurred in gels cultured during the same time. The increase in cell number in day 7-11 wounds was also significantly different from the increase in day 1-5 wounds (ANOVA, $P < 0.01$). The first-order rate constants for cell proliferation are presented in Table 2. Proliferation did not differ statistically between the controls for day 1-5 and for day 7-11 experiments.

Traction estimation. The resulting best-fit values for $r_0$ are presented in Table 3 for the various experimental conditions. RDFs implanted in day 1-5 wounds had a 30% higher traction than those from gels simultaneously maintained in culture. However, RDFs implanted in day 7-11 wounds exhibited 27% lower trac-
tion then those maintained in culture over the same period. Only one estimation of τ₀ could be determined for each condition because all gel compaction values were reduced to one compaction curve for each condition. Therefore, no statistical comparison of τ₀ could be performed.

Cell morphology. Images of typical cells from both the control and the implanted gels are presented in Fig. 7. While cell morphology in the gels was variable, these cells are representative of the predominant morphology present under each set of conditions. The cells from the implanted gels exhibited an elongated, polar morphology in both the day 1–5 and the day 7–11 wounds while the cells from cultured gels were typically stellate.

Actin staining. Typical results for α-smooth muscle actin and F-actin staining are presented in Fig. 8. In gels maintained in culture, more than half of the cells expressed α-smooth muscle actin. In the implanted gels from both the 1- to 5- and the 7- to 11-day periods there were almost no cells expressing α-smooth muscle actin.

Mechanical testing. Ultimate tensile strength measurements for implanted and control gels are presented in Table 4. There was no difference in ultimate tensile strength between gels maintained in culture and those implanted in the rat for 4 days.

**DISCUSSION**

Methods for a novel implantable collagen gel assay for fibroblast proliferation and traction have been presented. Fibroblast-populated collagen gels contained within a PVDF semipermeable membrane were placed within an implanted CV sponge at selected times following the initial wounding. Gels were subsequently removed and both the gel diameter and the cell number determined. Gel compaction of and cell proliferation in implanted gels populated by dermal fibroblasts were different from those of gels maintained in culture. Compaction and proliferation results were also different depending on the time frame during which the gels were placed in the wound site.

The rate of gel compaction is a function of both the cell number and the per-cell traction if the mechanical properties of the gel are constant. In our various in vitro assays for cell traction, cell proliferation rates have not varied significantly under the experimental conditions. As a result, for a given cell concentration, the differences in gel compaction can be tied directly to differences in cell traction [8, 11]. In this study, how-

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**TABLE 1**

<table>
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<th>Biphasic Theory Model Parameters</th>
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<tbody>
<tr>
<td>Parameter</td>
</tr>
<tr>
<td>Collagen network</td>
</tr>
<tr>
<td>Shear modulus, G (dyne/cm²)</td>
</tr>
<tr>
<td>Shear viscosity, η (dyne·s/cm²)</td>
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<tr>
<td>Drag coefficient, a₀ (dyne·s/cm²)</td>
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<tr>
<td>Poisson's ratio, ν (dimensionless)</td>
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<tr>
<td>Fibroblasts</td>
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<tr>
<td>Migration coefficient, D₀ (cm²/s)</td>
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<tr>
<td>Spreading steepness, kₛ (dimensionless)</td>
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<tr>
<td>Spreading half time, t₁/₂ (h)</td>
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ever, we observed large differences in the cell proliferation rates depending upon the time frame postwounding during which the gel was implanted. This is not unexpected since the wound site contains a plethora of growth factors with varying mitogenic potential whose concentrations change over the course of the wound healing response [12, 35–38]. To obtain an accurate estimate of cell traction in our implantable assay, we accounted for a variable cell proliferation rate (i.e., different values of $k_0$ for the two wound periods studied).

The proliferation rate constants for cells in the cultured gels were comparatively low (Table 2). This is consistent with previous observations that fibroblast proliferation is depressed in collagen gels [39]. The wound environment is replete with growth factors such as PDGF that are highly mitogenic for fibroblasts [1], so it is not surprising that we observed increased proliferation in the implanted gels. The significantly higher proliferation rate in day 7–11 wounds versus day 1–5 wounds (ANOVA, $P < 0.05$) is attributed to the time-varying composition of the wound environment. Various growth factors have been shown to have temporally varying concentrations in the wound [12, 37, 38]. It is likely that the concentrations and combinations of growth factors present during the 7- to 11-day period in our assay are simply more mitogenic for fibroblasts than the concentrations and combinations present during the 1- to 5-day period. Such temporal variation in growth factor concentration helps drive the wound healing response through its various phases.

In the day 1–5 wounds, the implanted gels compacted more rapidly than those maintained in culture (Fig. 5A). If not for the difference in cell proliferation between the two experimental conditions (Fig. 6A), this difference in compaction rate for gels that have similar mechanical properties (Table 4) would lead us to conclude that the fibroblasts exposed to the day 1–5 wound environment exhibited higher traction than their counterparts maintained in culture. However, without the use of the biphasic theory for cell-matrix mechanical interactions that allows us to account for the differ-

<table>
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<tr>
<th>Conditions</th>
<th>$k_0$ (s$^{-1}) \times 10^7$</th>
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<tbody>
<tr>
<td>Days 1 to 5</td>
<td></td>
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<tr>
<td>Maintained in culture</td>
<td>0.76 ± 0.30</td>
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<tr>
<td>Implanted in rat</td>
<td>9.92 ± 0.27</td>
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<tr>
<td>Days 7 to 11</td>
<td></td>
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<tr>
<td>Maintained in culture</td>
<td>1.76 ± 0.38</td>
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<tr>
<td>Implanted in rat</td>
<td>31.83 ± 3.41</td>
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</tbody>
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**TABLE 3**

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<tr>
<th>Conditions</th>
<th>$n_0$ (dyn cm/cell) $\times 10^2$</th>
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<tbody>
<tr>
<td>Days 1 to 5</td>
<td></td>
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<tr>
<td>Maintained in culture</td>
<td>1.57 ± 0.07</td>
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<tr>
<td>Implanted in rat</td>
<td>2.05 ± 0.10</td>
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<tr>
<td>Days 7 to 11</td>
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<tr>
<td>Maintained in culture</td>
<td>1.62 ± 0.06</td>
</tr>
<tr>
<td>Implanted in rat</td>
<td>1.18 ± 0.10</td>
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In our cultured collagen gels exhibited both typical fibroblast and myofibroblast phenotypes. However, hardly any myofibroblasts were present in our implanted gels. It appears that the soluble wound environment of both the 1- to 5- and the 7- to 11-day periods down-regulated α-smooth muscle actin expression and inhibited the myofibroblastic phenotype.

Traction is necessary for cell migration and restructuring of surrounding tissue fibers although the amount of traction required for both may differ [45]. Given that fibroblast migration and tissue restructuring are both crucial elements of the wound healing response, it is reasonable to expect that RDF traction would vary depending on the stage of wound healing. We have previously reported that PDGF BB increases the traction of RDFs in free-floating (stress-free) collagen gels in vitro [11]. It is also well known that the various PDGF isoforms are abundant in the early wound environment [46], though our own analysis of rat wound fluid has indicated that the PDGF BB concentration in the early wound is about an order of magnitude lower than that needed to produce an optimal response in our in vitro assays (unpublished data).

Since our implantable collagen gel was free-floating (i.e., it does not adhere to the PVDF membrane), the increase in RDF traction that we report herein for RDF implanted in day 1–5 wounds is consistent with our in vitro findings, especially if PDGF is one of the primary modulators of RDF traction in the early wound. Further, our in vitro studies revealed that the increase in RDF traction upon PDGF BB stimulation was correlated with an increase in fibroblast random migration [11]. While we are unable to directly measure migration in the implanted collagen gel, we can observe the cell morphology upon gel harvest. RDFs in implanted gels from both time periods tended to have an elongated, bipolar morphology.
very similar to that observed in the implanted gels while cells with a stellate morphology tend not to migrate [8]. As noted above, the lack of $\alpha$-smooth muscle actin expression in the implanted gels is also consistent with a migratory cell phenotype. Taken together, these observations suggest that cells in the implanted gels are stimulated to migrate by the growth factors present in the day 1-5 and 7-11 wound environments.

It is more difficult to interpret the observed decrease in traction during the 7- to 11-day period. During wound healing, fibroblasts sequentially migrate into the wound site, proliferate, synthesize ECM, remodel the tissue through matrix degradation and tractional restructuring (sometimes leading to wound contraction), and eventually recede from the wound site [1]. The duration of each of these steps in the sequence depends upon the nature of the wound, the species of the animal, and the unique biology of the individual. Moreover, the CV sponge itself can affect the time course of wound healing. Sponge size, composition, and porosity have been shown to affect the rate of granulation tissue formation in the sponge model [47] and, in our experience, the biological properties of wound fluid harvested from such sponges (unpublished observations). So, while it is reasonable to conclude that during days 1-5 of the wound healing response fibroblasts were in the process of migrating into the wound site and proliferating, it is less obvious what their predominant activity was during the day 7-11 period. Specifically, it is not clear in our assay whether fibroblasts have yet entered the matrix formation and remodeling phase of wound healing. Therefore, it is difficult to correlate the observed RDF traction with some specific wound healing behavior. A histological analysis of the wound and the determination of the levels of key growth factors in wound fluid taken from that time period might help put the observed decrease in traction in context. However, the high rate of proliferation seen during the 7- to 11-day period (Table 2) suggests that the fibroblasts implanted in the wound were still primarily engaged in the proliferative phase of the wound healing response.

It should be noted that our in vitro studies indicate that the mechanical stress state of the gel modulates RDF response to PDGF BB stimulation [11]. Others have observed morphological and metabolic differences
in cells depending on the stress state of the matrix [44, 48–50]. As a mechanically constrained tissue that has undergone platelet retraction and perhaps some amount of wound contraction, the provisional matrix of the early wound is arguably more like a stressed collagen gel than the unstressed one used in our implantable assay. Given that the stress state of the ECM is known to affect cellular behavior, differences between the stress state of our implantable gel and the wound tissue itself must be considered when interpreting the results.

A pure collagen gel is quite different from the provisional wound matrix of fibrin, collagen, fibronectin, and other matrix molecules [1]. It is well known that the ECM mediates cell phenotype and modulates responses to growth factors [1, 51, 52]. Normally, signals from early wound fluid would be combined with signals from the predominantly fibrin provisional wound matrix [1]. Therefore, the cells in our assay, because they are seeded in collagen, may be receiving abnormal cues for a wound environment. So, both the stress state and the composition of the gel used in these studies may diminish the physiological relevance of our results.

While the implantable free-floating collagen gel is clearly not a direct analog of wound tissue, it does provide a defined, reproducible, three-dimensional biological reference matrix for studying cellular behavior. The assay allows us to study the effect of the soluble wound environment on cells in the reference matrix, and more importantly, it also allows us to study how the effect of the soluble wound environment changes over the course of the wound healing response. Further, we are exploring ways to create a version of the assay that includes a mechanical constraint on compaction that would allow us to investigate the role of mechanical stress on fibroblast behavior in vivo. Additionally, we could use fibrin gel in place of collagen, but attempts to prevent fibrin from adhering strongly to the PVDF membrane, thereby preventing gel compaction, have not yet been successful. Similarly, fibrinolysis might alter the mechanical properties of the gel and complicate the use of our theory for cell-matrix mechanical interactions to infer cell traction from gel compaction.

Because of the differences in cell proliferation rates observed under our various experimental conditions, we have used the biphasic theory for cell-matrix mechanical interactions to estimate cell traction. In doing so, we have made implicit assumptions about the mechanical properties of the collagen gel. Shear [30] and compression [31] experiments have shown the collagen network to exhibit viscoelastic fluid behavior. For simplicity, the network is modeled as a single relaxation time compressible Maxwell fluid [29]. This assumption has been shown to yield fairly accurate results for cell compaction of collagen gels up to about a 15% strain [53]. It is clear from Fig. 5 that the implantable gels experience strains of nearly 50% at the later time points. At these higher strains, the model fit becomes poorer and the resulting estimate of $\tau_0$ is less accurate. However, we are still able to undertake a comparative analysis of $\tau_0$ and can conclude that the traction exerted by cells implanted in day 1–5 wounds is greater than that of those maintained in culture and those implanted in day 7–11 wounds.

Our ability to perform a comparative analysis of cell traction rests upon the assumption that the mechanical properties of the collagen network do not differ significantly from one gel to another. We have established in vitro that the gel properties are relatively constant if the gels are formed under the same conditions [31]. However, in vivo we introduce additional variables. Specifically, we must consider the possible effects of proteinases present during wound healing that may degrade the collagen and alter the network properties. Our implantable gel is composed of type I collagen, and a group of matrix metalloproteinases that degrade collagen, the collagenases, is found in the wound [54].

To attempt to assess possible degradation, we mechanically tested several gels in uniaxial extension. We compared the ultimate tensile strength of implanted gels to that of those maintained in culture. As shown in Table 4 there was no significant difference. Due to the small size and relatively weak nature of our gels, accurate measurement of the ultimate tensile strength was difficult. The load at failure was only about three to four times the force resolution limit for the mechanical test system, so small changes in the gel mechanical properties due to collagen degradation were not detectable. Nevertheless, the ultimate tensile strength measurements provided no evidence of large differences in terms of mechanical properties between gels maintained in culture and those implanted in wounds that might invalidate the assumptions of the biphasic theory. Degradation of the matrix and the resulting change in mechanical properties may be a more significant concern if the gels are exposed to the wound environment for longer periods of time or implanted at later time points such as during the tissue remodeling phase when collagenase activity may be higher.

This assay combines the more quantitative nature of in vitro assays with the greater physiological relevance of in vivo wound healing models. Cells are exposed to the soluble wound environment but otherwise isolated from the host animal. As the cells are sequestered within the PVDF dialysis membrane, they can be recovered from the wound at prescribed time points. We have demonstrated herein the ability to quantify fibroblast proliferation and traction upon exposure to the soluble wound environment using the assay. There are, however, a number of other ways in which the unique
features of this assay could be employed. Though we have not done so, wound fluid could also be harvested from the CV sponge and its composition analyzed and correlated with the time varying cell behavior. Syngeneic cells other than dermal fibroblasts could be implanted. Endothelial cells have been shown to form tubes in three-dimensional collagen gels in vitro [55]; the effect of the soluble wound environment on this behavior could readily be studied using our assay. Genetically modified cells that are unresponsive to certain growth factors could be implanted and the assay used to assess the role of these growth factors in modulating cell behavior in wound healing. Using the dominant negative receptor technique [56], we are currently developing a fibroblast that lacks signaling capability through the PDGF β receptor so that we can probe the role of PDGF BB in regulating fibroblast traction and proliferation in the wound. Herein, we have used immunocytochemical techniques to study the expression of α-smooth muscle actin by cells in implanted gels. Similar methods could be used to probe for the expression of other proteins. Alternatively, mRNA levels could be measured to assess gene expression.

**APPENDIX**

The biphasic theory for cell–matrix mechanical interactions has been described in detail elsewhere [28, 29]. We provide a brief overview here of the isotropic version of the theory for the assay presented.

The model consists of five conservation equations and two constitutive equations. The mass conservation equations for the network and solution phases in terms of volume fractions $\theta_n$ and $\theta_s$ are

$$\frac{D\theta_n}{Dt} + \theta_n(\nabla \cdot \mathbf{v}_n) = 0,$$

$$\frac{D\theta_s}{Dt} + \theta_s(\nabla \cdot \mathbf{v}_s) = 0,$$

where $\mathbf{v}_n$ and $\mathbf{v}_s$ are the velocities of the phases and $D/Dt$ denotes the substantial derivative moving with each phase. The momentum conservation equations for the solution and network are

$$\nabla \cdot [\theta_s(\mathbf{P} \mathbf{I})] + \phi(\mathbf{v}_s - \mathbf{v}_n) = 0,$$

$$\nabla \cdot [\theta_n(\sigma + c\tau_0 \mathbf{I} - \mathbf{P} \mathbf{I})] + \phi(\mathbf{v}_s - \mathbf{v}_n) = 0,$$

where $P$ is the hydrostatic pressure, $\phi_0$ is an interstitial drag coefficient between the network and the solution phases, $\sigma$ is the network viscoelastic stress tensor, and $\tau_0$ is the cell traction parameter.

The cell conservation equation accounting for cell proliferation, convection, and migration is

$$\frac{Dc}{Dt} + c(\nabla \cdot \mathbf{v}_n) = D_0 \nabla^2 c + k_0 c,$$

where $c$ is the cell concentration, $D_0$ is the cell migration coefficient, and $k_0$ is the first-order rate constant for cell proliferation. Since the cells are considered a species in the network phase (in which they are convected and migrate and must attach for proliferation), the cell concentration is defined per unit volume of network phase and the substantial derivative is with respect to $\mathbf{v}_n$.

The network was modeled using the single relaxation time compressible Maxwell fluid equation, which has been shown to be of sufficient accuracy for compacting gels up to about 15% strain [30, 53],

$$\frac{1}{2G}\dot{\sigma} + \frac{1}{2\eta} \sigma = \frac{1}{2} [\nabla \mathbf{v}_n + (\nabla \mathbf{v}_n)^T] + \frac{v}{1-2v} (\nabla \cdot \mathbf{v}_n) \mathbf{I},$$

where $G$ is the shear modulus, $\eta$ is the shear viscosity, and $v$ is Poisson’s ratio. The dot denotes the upper convected derivative.

In order to account for cell spreading and development of motility from an initial nonmotile state when the gel is formed, the cell migration and traction stress coefficients were modified accordingly with a sigmoidal dependence on time [28, 30],

$$D_0(t) = D_0 \frac{t^{k_{\infty}}}{(t^{k_{\infty}} + (t_{1/2})^{k_{\infty}})},$$

$$\tau_0(t) = \tau_0 \frac{t^{k_{\infty}}}{(t^{k_{\infty}} + (t_{1/2})^{k_{\infty}})},$$

where $k_{\infty}$ determines the steepness of the spreading vs time response and $t_{1/2}$ is the half time for cell spreading.

For the case of the cylindrical collagen gel used to model our implantable assay, the boundary conditions for the symmetry plane and axis of the cylinder are

$$\mathbf{v}_n = (\sigma + \tau_0 \mathbf{I})_n = \frac{\partial c}{\partial n} = \frac{\partial P}{\partial n} = 0,$$

where $v$ is a component of the network velocity and $n$ and $t$ refer to the normal and tangent directions. The boundary conditions for the surfaces are

$$P = (\sigma + \tau_0 \mathbf{I})_{nt} = (\sigma + \tau_0 \mathbf{I})_n = \frac{\partial c}{\partial n} = 0.$$

$\tau_0$ is estimated by fitting the gel compaction data (network displacement data) to the model equations using a finite element method to discretize the spatial deriv-
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