Magnitude and Duration of Stretch Modulate Fibroblast Remodeling

Jenna L. Balestrini
Kristen L. Billiar

Departments of Biomedical Engineering and Mechanical Engineering, Worcester Polytechnic University, Worcester, MA 01609-2280; Department of Mechanical Engineering, Department of Surgery, University of Massachusetts Medical School, Worcester, MA 01655

1 Introduction

Cells within skin and other connective tissues are continuously subjected to a range of mechanical forces from external loading and cell-generated tension. These mechanical cues guide fibroblast-mediated tissue remodeling and ultimately regulate the structure of a stable and well-structured extracellular matrix (ECM) optimized to resist these loads [1]. When the ECM is disrupted due to injury, the loads applied to the cells are substantially altered, which, in turn, leads to an increase in cell-generated forces and remodeling of the tissue architecture. The increase in cell-generated tension results in the alignment of the collagen-rich ECM along these lines of tension, creating scar tissue with lower strength, compliance, and capacity to withstand multiaxial loads as compared with normal tissue. In addition, this increase in cell-generated force creates the potential for contracture and deformity [2].

Stretching wounds during healing (e.g., range of motion exercises, splinting, and vacuum pressure) is used routinely by clinicians to attain improved tissue properties and to manipulate healing rates and may aid in obtaining a stable bond between the skin and percutaneous devices [2–4]. In experimental models, the use of mechanical stimulation during wound healing has been shown to increase collagen production and to decrease contracture of granulation tissue [5]. Alternatively, increased loading of the dermis is also associated with negative effects such as excessive collagen production and contracture, common processes found in many fibrocontractive diseases [6–8]. Determining loading regimens that promote the desired aspects of healing without stimulating detrimental side effects would be of obvious benefit.

Similar to observations in vivo, researchers have demonstrated that mechanical conditioning tissue models in vitro results in stronger, stiffer tissue as compared with statically cultured controls [8–15]. The most common three-dimensional (3D) tissue models utilized for mechanobiological studies are cell-populated collagen and fibrin gels [16]. Collagen gels have been extensively utilized as tissue models to investigate the interplay between tissue mechanics, cell-matrix interactions, and mechanobiology in a controlled in vitro manner as they allow for precise control over culture conditions, specimen composition, and boundary conditions [14,17–19]. These connective tissue analogs form the dermal component of clinically available tissue engineered skin replacements, yet they lack sufficient mechanical integrity and durability for clinical usage in load-bearing applications [20]. While fibrin gels were initially developed to model the early stages of connective tissue healing (fibroplasia) for mechanobiological studies, these systems have recently emerged for use as tissue engineering scaffolds. Fibrin gels are initially very weak but, unlike collagen gels, do not inhibit ECM production and achieve tissue-like strength following sufficient synthesis and assembly of collagen [9,20–25]. These systems therefore have a strong potential for use as tissue replacements in wound healing therapy and regenerative medicine, and also for restoring functionality to damaged or diseased tissue [21,24,26]. Although much has been learned about the mechanisms of strain-dependent remodeling by utilizing collagen and fibrin gels [10,25,27], the combinations of strain levels, ranges, and durations utilized thus far are not sufficient to charac-
terize the complex relationships between mechanical loading pa-
rameters (magnitude, duration, etc.) and remodeling parameters
(strength, stiffness, alignment, etc.). Quantitative dose-response
curves would aid in the rational design of therapies and would
assist in the understanding and prevention of scarring.

The goal of this investigation is to quantitatively study the re-
lationship between stretch magnitude and duration on the changes
in matrix mechanics and composition in a 3D planar model of
wound healing. The present work follows a previous study where
we observed that cyclic equibiaxial stretch at 16% for 24 h/day
dramatically increases the collagen content and tissue strength of
fibroblast-populated fibrin gels [13]. In this study, these tissue
models are cycled as little as 2% and as much as 16% equibiaxi-
ally for 6 h/day or 24 h/day. We hypothesize that the extent of
stretch and the number of hours of stretch per day. This work will
extend our knowledge of how mechanical cues can be used to
manipulate key properties of healing wounds and will also con-
tribute valuable information for the creation of custom tailored
tissue equivalents based on cell-populated fibrin gels.

2 Materials and Methods

2.1 Fabrication of Fibrin Gels. Fibroblast-populated fibrin
gels were produced as previously described [13]. Briefly, bovine
fibrogenin and thrombin (Sigma, St. Louis, MO) were combined
with passage 7–9 human foreskin fibroblasts (American Type Cul-
ture Collection, Manassas, VA) in Dulbecco’sModified Eagle Me-
dium (DMEM, Mediatech, Herndon, VA) supplemented with 10%
bovine calf serum (BSC, HyClone, Logan, UT), 100 U/ml pen-
cillin G sodium, 100 μg/ml streptomycin sulfate, and 250 ng/ml
amphoteratin B (Gibco, Grand Island, NY). The final concen-
trations of the constituents were 0.2 U/ml thrombin, 3.8 mg/ml fi-
brinogen (Sigma, St. Louis, MO), and 500,000 cells/ml. The fibrin
and cell solution (3 ml) was poured into six 35 mm diameter
untreated flexible-bottom culture wells with circular fibrous foam
anchors (Flexcell International, Hillsborough, NC) and allowed to
polymerize for 24 h prior to mechanical stimulation. The gels
were cultured at 37 °C with humidified 10% CO2. DMEM supple-
mented with 10% fetal bovine serum (FBS, HyClone), 25 g/l
L-ascorbate (Wako, Richmond, VA), 0.5 ml/l insulin (Sigma), 100
U/ml penicillin G sodium, 100 μg/ml streptomycin sulfate, and
250 ng/ml amphoteratin B (Gibco) was changed every other day.

2.2 Application of Stretch. A vacuum-driven loading device
(FX-4000T, Flexcell International) was utilized to apply uniform
equibiaxial cyclic stretch to the fibrin gels as previously described
[13]. The gels were cultured statically (control) or subjected to
either continuous stretch (24 h/day) or intermittent stretch (6
h/day) with a sinusoidal waveform at 2%, 4%, 8%, or 16% equibi-
axial stretch at a frequency of 0.2 Hz for 8 days. One limitation of
the standard Flexcell system is that only one magnitude of stretch
can be attained at any given time. Therefore, in the continuously
stretched group, there is an individual set of parallel controls for
each treatment group (i.e., each stretch magnitude); data from
samples in the continuously stretched groups were normalized
with respect to the parallel statically cultured controls. At the con-
clusion of each experiment, samples were processed for biochemi-
cal, histological, or mechanical testing as described in Secs.
2.3–2.7.

To apply intermittent stretch, gels were cyclically stretched for
6 h/day. The six-well plates were then removed from the stretch
device and cultured statically for the remaining 18 h of each day
for 8 days. The same stretch magnitudes and frequency were used
for the intermittently stretched fibrin gels as the continuously
stretched gels. At the conclusion of each experiment, the gels were
characterized identically to the continuously stretched gels. In ad-
dition, subfailure biaxial testing was performed on a subset of the
samples from this group as described in Sec. 2.5. Since each of
the four groups is cultured only 6 h each day, all four magnitudes
were performed in parallel with one statically cultured control
group.

2.3 Determination of Cell Number and Total Collagen
Content. Cell number was determined by liberating the cells from
the inner 25 mm diameter area of the fibrin gels by treating the
gels with 0.05% trypsin (Gibco) and 2 mg/ml type I collagenase
(Sigma) for 30 min at 37 °C. Samples (refer to Tables 2 and 3 for
sample sizes) were stained with trypan blue and the cell number
was quantified using a hemocytometer. Collagen content was
quantified by the hydroxyproline assay [28]. The amount of
collagen was estimated using a conversion of 0.13 g of hydroxypro-
line per gram of collagen [29], and the accuracy of each assay was
verified with a 1 mg/ml collagen standard (Sirlco, Biocolor, West-
bury, NY). Collagen density was quantified as the dry weight of
collagen per volume of each gel, as determined by multiplying
measured thickness (described below) by the area of inner 25 mm
diameter section removed from the anchors for analysis.

2.4 Determination of Physical Properties. The thickness, ul-
timate tensile strength (UTS), failure tension, and extensibility of
the samples were calculated as described in detail previously [13]
and outlined briefly below. To eliminate active tissue retraction
prior to testing, the actin-rich cytoskeletons of the fibroblasts were
depolymerized by treating all of the samples with 6 μM cytochal-
asin D (Sigma) for four hours. Following the cytochalasin D
treatment, the central 25 mm section of each fibrin gel was cut
from the anchors and carefully lifted from the flexible-bottom
membranes.

To perform a compaction assay, a standard method to measure
the capacity of fibroblasts to remodel ECM components [30],
sample thickness was quantified by placing a small reflective disk
(1.3 g, 13 mm diameter) onto the centermost region of the sample
and allowing the tissue to reach equilibrium. The height was re-
corded using a laser displacement system (LDS) (±10 μm, LK-
081, Keyence, Woodcliff Lake, NJ). Using a custom equibiaxial
tissue inflation system [31], the tissue sample was circularly clamped and the centermost 5 mm section was inflated with iso-
tonic room temperature saline until burst. The displacement was
measured at the center of the sample by the LDS, and pressure
during inflation was measured by a pressure transducer (+0.13 kPa). The radius of curvature at failure, R, was deter-
rmined from the sample height assuming a spherical cap geometry
(validated in Ref. [31]). The tension at failure, T, was then deter-
mined using the Law of Laplace, T=1/2PR, where P is the burst
pressure. The UTS was defined as the tension at failure divided by
the undeformed thickness of the fibrin gel (as measured by the
LDS). The extensibility was defined as the (equibiaxial) Green’s
strain at the center of the sample at the failure pressure [32]. The
sample numbers for each treatment group are given in Tables 2
and 3.

2.5 Low-Force Biaxial Mechanical Characterization. To
investigate the effects of stretch magnitude on matrix stiffness,
Young’s modulus of a subset of the intermittently stretched fibrin
gels was determined using planar biaxial testing. Samples were
removed from their silicone substrates and the thickness was mea-
ured with the LDS (as described above). To ensure the stiffness
measurements would not be affected by the foam anchor, the
anchor was cut radially into 20 equal sections from the fibrin-foam
anchor interface to the outer edge of the anchor. To attach the
tsamples to the device, 16 stainless steel hooks (four on each “side”
of the sample) were attached to the fibrous foam anchor sections
surrounding each sample; the four “corner” segments were not
utilized as indicated by blank spaces around the edge of the
sample in Fig. 1. The sample was placed in a temperature-
controlled, isometric saline-filled bath (maintained at 37 °C), and
the hooks were attached with nylon suture to a dual pulley system,
which distributes equal force to each of the four hooks on each

Transactions of the ASME

051008-2 / Vol. 131, MAY 2009
side of the sample. Each pulley system was attached to an actuator by a 7.6 cm plexiglass arm extending out of the bath either directly or via a torque transducer (0.15 N m, Futek, Irvine, CA), which was used to measure force with ±4 mN accuracy. Small Styrofoam floats were placed at the base of each hook to maintain buoyancy of the sample.

The actuators were drawn apart until the sample was restored to the original outer dimensions, and four small marker chips were affixed on the central region of the sample to form a 49 mm² region. Digital images were acquired using an analog video camera (XC-ST50, Sony, Park Ridge, NJ) and an image acquisition board (PCI-1405, National Instruments, Austin, TX), and a custom video marker-tracking algorithm was used to track the markers. A subregion was automatically positioned on each marker, and displacements were calculated using LABVIEW (National Instruments). Each specimen was preconditioned with 10 cycles to 10% equibiaxial engineering strain quasistatically at a strain rate of 0.01 s⁻¹. Engineering stress was calculated by dividing the force by the cross-sectional area of the tissue. The cross-sectional area of the tissue was measured by multiplying the sample width by the sample thickness. Young’s modulus for each sample was determined by fitting the data from the final equibiaxial protocol to the equation for a homogeneous linear elastic solid using MATLAB (Mathworks, Inc., Natick, MA) software. The structural stiffness was calculated by multiplying Young’s modulus by the thickness of the sample.

2.6 Retraction Assay. After the total culture period of 9 days, a subset of fibrin gels from each group was gently released from the actuators which was used to measure force with a torque transducer (0.15 N m, Futek, Irvine, CA) and an image acquisition board (PCI-1405, National Instruments, Austin, TX), and a custom video marker-tracking algorithm was used to track the markers. A subregion was automatically positioned on each marker, and displacements were calculated using LABVIEW (National Instruments). Each specimen was preconditioned with 10 cycles to 10% equibiaxial engineering strain quasistatically at a strain rate of 0.01 s⁻¹. Engineering stress was calculated by dividing the force by the cross-sectional area of the tissue. The cross-sectional area of the tissue was measured by multiplying the sample width by the sample thickness. Young’s modulus for each sample was determined by fitting the data from the final equibiaxial protocol to the equation for a homogeneous linear elastic solid using MATLAB (Mathworks, Inc., Natick, MA) software. The structural stiffness was calculated by multiplying Young’s modulus by the thickness of the sample.

The optimal parameter values ($R_0, R_0, \tau$) were determined using MATLAB (Mathworks, Inc.) by minimization of least-squares error.

To ensure that our calculated value of the intercept, $R_0$, represents an accurate measurement of the passive component of the total retraction, a separate subset of samples was treated with 6 μM cytochalasin D (for 4 h), allowed to retract, and the resulting percent retraction compared with the calculated value of $R_0$ (data not shown). As passive retraction is not a common engineering metric for remodeling, we also calculate the residual strain, or effective prestrain, in the matrix using the following equation:

$$e_{res} = \frac{(d_f - d_i)}{d_i}$$

where the initial diameter ($d_i$) is defined as the dimension following release of prestress (calculated from the fit parameter $R_0$ above), and the final diameter ($d_f$=25 mm) is defined as the diameter of the sample when attached to the anchor (with residual stress).

2.7 Histological Analysis. Representative samples from each group were fixed in buffered formalin while still attached to the anchors for 18–20 h at 4°C and stored in 70% ethanol for histological evaluation. The samples were then removed from their anchors and silicone membranes, embedded in paraffin, cut into 4 μm sections, and then stained with hematoxylin and eosin (H&E). Micrographs were taken with a Nikon Eclipse E600 camera at a magnification of 200×.

2.8 Statistical and Regression Analyses. Data are presented as mean ± standard deviation. For each treatment group, all remodeling metrics were normalized to the respective statically cultured control groups. To determine trends in remodeling metrics, the normalized data were first analyzed using a two-way analysis of variance (ANOVA) (SigmaStat, Systat Inc., San Jose, CA) to isolate factors with statistically significant effects and power. The factors analyzed in the ANOVA were stretch magnitude ($M$), duration of stretch per day ($t$) set to 0 for 24 h/day and 1 for 6 h/day), and the interaction term ($I$) between these factors (defined as the magnitude factor times the duration factor). If factors were found to have sufficient statistical significance ($p<0.05$) and power ($≥0.8$) in the ANOVA, they were further analyzed for trends using regression analysis (SAS Institute Inc., Cary, NC). The coef-
efficient of determination ($R^2$) and the root mean square (RMS) error were used to assess the goodness of fit to the models.

3 Results

3.1 Effect of Stretch on Compaction. After 9 days in culture, the fibroblasts rapidly condensed and reorganized the fibrin matrix in all groups as seen in the histomicrographs (Fig. 2). By gross observation, all gels appeared relatively uniform in thickness across the membrane (including the fibrin-anchor interface), and all cycled gels appeared less transparent and less hydrated than their statically cultured controls. Based on thickness measurements, both continuously stretched and intermittently stretched gels compacted in a dose-dependent manner with increasing stretch, although the trends of compaction did differ slightly between these groups. In combination, compaction was linearly correlated with stretch magnitude with no significant correlation to the rest period or interactive term. Table 1 provides the regression models for compaction and other remodeling parameters as a function of all stretch factors, and Tables 2 and 3 list the raw data for each treatment group. Figure 3 represents these data graphically along with biochemical and mechanical data described in Secs. 3.2–3.5.

3.2 Effect of Stretch on Mechanical Properties. All cycled gels were stronger than their respective controls in terms of both UTS and failure tension. There was an exponential increase in UTS with increasing stretch magnitude. Intermittently stretched gels in general had a larger UTS than continuously stretched gels (e.g., 35% stronger at 8% stretch and 26% at 16% stretch), but the impact of a rest period was not statistically significant (Fig. 3, Table 1). In contrast, for failure tension there was a linear dependence on both stretch magnitude and the interaction between stretch magnitude and the rest period (Table 1). Specifically, the failure tension increased with increasing stretch magnitude in the intermittently stretched gels (Fig. 1, Table 3) whereas the failure tension increased uniformly at all stretch magnitudes in gels cycled continuously (13–17% greater tension at failure relative to respective controls, Table 2). The extensibility increased with increasing stretch magnitude in the intermittent stretch group; no trend in extensibility with magnitude was observed in the continuously stretched group.

3.3 Effect of Stretch on Cell Number and Collagen Density. There was no change in cell number for gels stretched continuously in at any stretch level (Fig. 3). However, when gels

---

### Table 1 Regression analysis for normalized remodeling metrics as a function of stretch magnitude ($M$, the length per day of stretch (CS versus IS), and an interaction term ($I$, see statistical analysis methods). The fibroblast-populated fibrin gels were stretched continuously or intermittently at 0%, 2%, 4%, 8%, or 16% stretch for 8 days at 0.2 Hz. Note that although the passive retraction was determined to be significantly impacted by magnitude, and cell number was determined to be significantly impacted by both magnitude and the interaction term, they did not follow simple linear, exponential (exp), or logarithmic regression models and are therefore not listed in the table.

<table>
<thead>
<tr>
<th>Remodeling Metric</th>
<th>Regression Model</th>
<th>$R^2$</th>
<th>RMS Error</th>
</tr>
</thead>
<tbody>
<tr>
<td>Thickness</td>
<td>0.99 - 0.06(M)</td>
<td>0.91</td>
<td>0.12</td>
</tr>
<tr>
<td>UTS</td>
<td>1.77 + (1.60 * 10^6)expM</td>
<td>0.91</td>
<td>1.88</td>
</tr>
<tr>
<td>Failure Tension</td>
<td>1.05 + 0.06(M) - 0.05(I)</td>
<td>0.78</td>
<td>0.15</td>
</tr>
<tr>
<td>Collagen Density</td>
<td>2.23 + (4.90<em>10^6)expM - (2.81</em>10^6)expI</td>
<td>0.93</td>
<td>3.41</td>
</tr>
</tbody>
</table>

### Table 2 Raw mechanical, biochemical, and physiological data for continuously stretched gels cycled at 0%, 2%, 4%, 8%, and 16% stretch magnitudes for 8 days at 0.2 Hz. Each continuously stretched group was run with a separate statically cultured control group, thus the number of samples in the “0” (static) group is very large.

<table>
<thead>
<tr>
<th>% Stretch (Continuous)</th>
<th>Thickness (mm)</th>
<th>[n]</th>
<th>UTS (kPa)</th>
<th>Failure Tension (N/m)</th>
<th>Extensibility</th>
<th>Collagen Density (g/cm³)</th>
<th>[n]</th>
<th>Cell Number (*10⁶ cells) [n]</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>1.29 ± 0.30</td>
<td>28</td>
<td>9.6 ± 3.0</td>
<td>11.3 ± 1.9</td>
<td>2.3 ± 0.4</td>
<td>0.13 ± 0.05</td>
<td>12</td>
<td>0.30 ± 0.18</td>
</tr>
<tr>
<td>2</td>
<td>1.15 ± 0.16</td>
<td>6</td>
<td>11.7 ± 1.8</td>
<td>13.3 ± 2.1</td>
<td>2.7 ± 0.5</td>
<td>0.09 ± 0.01</td>
<td>3</td>
<td>0.48 ± 0.07</td>
</tr>
<tr>
<td>4</td>
<td>0.76 ± 0.04</td>
<td>4</td>
<td>16.3 ± 2.2</td>
<td>12.5 ± 1.3</td>
<td>2.2 ± 0.5</td>
<td>0.20 ± 0.01</td>
<td>3</td>
<td>0.97 ± 0.04</td>
</tr>
<tr>
<td>8</td>
<td>0.42 ± 0.08</td>
<td>5</td>
<td>32.2 ± 4.6</td>
<td>15.5 ± 2.3</td>
<td>2.6 ± 0.6</td>
<td>0.49 ± 0.08</td>
<td>3</td>
<td>0.95 ± 0.07</td>
</tr>
<tr>
<td>16</td>
<td>0.13 ± 0.02</td>
<td>9</td>
<td>101.2 ± 21.2</td>
<td>13.2 ± 1.1</td>
<td>1.9 ± 0.4</td>
<td>2.91 ± 0.44</td>
<td>3</td>
<td>0.89 ± 0.17</td>
</tr>
</tbody>
</table>
were stretched intermittently the cell number increased with increasing stretch, and peaked at 16% stretch ($p < 0.001$). Collagen density increased exponentially as a function of stretch magnitude, and there was significant interaction between magnitude and the rest period (Table 1).

### Table 1

<table>
<thead>
<tr>
<th>% Stretch (Intermittent)</th>
<th>Thickness (mm)</th>
<th>[n]</th>
<th>UTS (kPa)</th>
<th>Failure Tension (N/m)</th>
<th>Extensibility</th>
<th>[n]</th>
<th>Collagen Density (g/cm²)</th>
<th>[n]</th>
<th>Cell Number (*10⁶ cells)</th>
<th>[n]</th>
<th>Young's Modulus (kPa)</th>
<th>Structural Stiffness (N/m)</th>
<th>[n]</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>1.53 ± 0.04</td>
<td>5</td>
<td>8.0 ± 2.3</td>
<td>12.2 ± 3.6</td>
<td>1.4 ± 0.5</td>
<td>4</td>
<td>0.16 ± 0.03</td>
<td>3</td>
<td>0.41 ± 0.21</td>
<td>2</td>
<td>7.2 ± 1.4</td>
<td>350 ± 72</td>
<td>3</td>
</tr>
<tr>
<td>2</td>
<td>1.60 ± 0.16</td>
<td>2</td>
<td>9.0 ± 2.8</td>
<td>14.3 ± 4.5</td>
<td>1.4 ± 1.0</td>
<td>2</td>
<td>0.22 ± 0.11</td>
<td>3</td>
<td>0.42 ± 0.01</td>
<td>2</td>
<td>5.1 ± 2.0</td>
<td>304 ± 140</td>
<td>2</td>
</tr>
<tr>
<td>4</td>
<td>1.05 ± 0.05</td>
<td>4</td>
<td>17.9 ± 3.0</td>
<td>18.6 ± 2.2</td>
<td>2.0 ± 0.2</td>
<td>4</td>
<td>0.32 ± 0.15</td>
<td>3</td>
<td>0.45 ± 0.13</td>
<td>2</td>
<td>14.2 ± 1.3</td>
<td>462 ± 103</td>
<td>2</td>
</tr>
<tr>
<td>8</td>
<td>0.39 ± 0.03</td>
<td>5</td>
<td>49.9 ± 8.7</td>
<td>19.1 ± 2.9</td>
<td>2.3 ± 0.2</td>
<td>5</td>
<td>1.67 ± 1.35</td>
<td>3</td>
<td>0.51 ± 0.33</td>
<td>2</td>
<td>65.5 ± 8.7</td>
<td>687 ± 149</td>
<td>2</td>
</tr>
<tr>
<td>16</td>
<td>0.18 ± 0.02</td>
<td>5</td>
<td>136.8 ± 14.2</td>
<td>24.1 ± 1.4</td>
<td>2.5 ± 0.1</td>
<td>5</td>
<td>7.96 ± 0.59</td>
<td>3</td>
<td>0.50 ± 0.16</td>
<td>2</td>
<td>57.3 ± 10.0</td>
<td>353 ± 58</td>
<td>3</td>
</tr>
</tbody>
</table>

#### 3.4 Effect of Stretch on Matrix Retraction

After release, all fibrin gels rapidly retracted due to a combination of prestress in the matrix and active cellular contraction, decreasing in projected area by approximately 60% or more within 10 min (e.g., see Fig. 4). Overall trends in retraction for gels cycled at 0%, 2%, 4%, 8%, and 16% stretch magnitudes for 8 days at 0.2 Hz, and normalized to statically cultured controls from each experiment. Note that UTS, stiffness, and collagen density are, by definition, directly dependent on the thickness, whereas the other parameters are independent of the degree of compaction. For clarity, statistical models are provided in Table 1.

![Fig. 3](image-url)
3.5 Effect of Intermittent Stretch on the Matrix Stiffness. The elastic modulus increased slightly from control to 2% and 4%, increased to a maximum at 8%, and then dipped slightly down at 16% with intermittent stretch (Fig. 5, Table 1). As biaxial tests were not performed on continuously stretched samples, the interaction between stretch magnitude and the rest period could not be determined.

4 Discussion

Establishing quantitative relationships between mechanical simulation and extracellular matrix remodeling is an important step toward rational design of manual therapies for wound healing and harnessing stretch as a means to custom tailor tissue analogs with specific requirements (e.g., strength, stiffness, and contractility). In this study, using multiple levels of equibiaxial stretch, we determined that tissue strength, stiffness, and the accumulation of collagen increased with increasing stretch magnitude. Furthermore, we discovered that while compaction and compaction-related remodeling (e.g., UTS) were highly dependent on stretch magnitude, stretch-induced increases in structural properties (e.g., failure tension) were contingent upon a rest period. These results demonstrate that the magnitude and the duration per day of stretch

and 16% strain were very similar between gels cycled continuously ($n=16, 2, 3, 3$, and $7$, respectively) and intermittently ($n=3, 3, 3, 3$, and $2$, respectively). Although stretch magnitude was determined to be a statistically significant parameter governing passive retraction ($R_p$) per the ANOVA, the data do not follow a simple trend (linear, exponential, or logarithmic). Furthermore, despite a consistent decrease in active cell-mediated matrix retraction ($R_a$) in the 2% stretched gels for both groups, there was no statistical dependence of this remodeling metric on the stretch magnitude or duration. The effective prestrain ($e_r$) values for the groups cycled continuously to 0%, 2%, 4%, 8%, and 16% were $42 \pm 17\%, 17 \pm 5\%, 29 \pm 1\%, 30 \pm 1\%, 13 \pm 4\%$, respectively, and the intermittently cycled groups were $10 \pm 2\%, 13 \pm 4\%, 9 \pm 3\%, 8 \pm 3\%$, and $4 \pm 1\%$, respectively.

The time constants of retraction for the groups cycled continuously to 0%, 2%, 4%, 8%, and 16% were $3 \pm 1$, $5 \pm 3$, $8 \pm 1$, $7 \pm 2$, and $7 \pm 4$, respectively. The time constants of retraction for the intermittently cycled groups to 0%, 2%, 4%, 8%, and 16% were $10 \pm 2$, $13 \pm 4$, $9 \pm 3$, $8 \pm 3$, and $4 \pm 2$, respectively. There was no correlation between stretch magnitude and the rate at which the matrices retracted, and there was no significant effect of the duration (6 h/day versus 24 h/day) on the rate of retraction.

Fig. 4 (a) Representative fibroblast-populated fibrin gel at 40 s and 7 min post release from its substrate. The gels are placed in a 35 mm tissue culture dish and are free floating in culture media. The dashed line represents the initial area of the fibrin gel (25 mm in diameter) that was dynamically cultured for 8 days and then cut away from its circumferential anchors. Note the rapid decrease in projected-sectional area. (b) Representative data of total matrix retraction as a function of time. The data are fitted to an exponential increase equation as represented by the dotted line with three parameters; $R_t$ (total retraction), $R_a$ (active retraction), $R_p$ (passive retraction), and $\tau$ (time constant), shown schematically in the figure. The bulk of the retraction occurs in less than 10 min ($\tau \sim 9$ min), and tensional homeostasis is reached by approximately 30 min.

Fig. 5 (a) Representative engineering stress-strain plot of equibiaxial loading along orthogonal “1” and “2” directions demonstrating isotropy of the matrix in a gel cycled intermittently at 4% strain for 8 days. Note that the 1 and 2 directions result in identical stress-strain profiles; this similarity is a result of the in-plane isotropy of the material. (b) Representative stress-strain data from gels stretched at 0%, 2%, 4%, 8%, and 16% stretch for 6 h a day at 0.2 Hz. Stiffness generally increases with increasing culture stretch magnitude, with a large increase between the 4% and 8% strain-conditioned groups, but no significant change between the 8% and 16% strain-conditioned groups.
are both critical parameters in matrix remodeling and suggest that these two factors can be used independently or in concert to manipulate different aspects of remodeling.

4.1 Cyclic Stretch Increases Tissue Strength in Fibrin Gels. The data from this study confirm our previous results and the findings of others that cyclically stretching cell-populated biomaterial gel results in significant increases in mechanical properties [1,8,10–13,19,21,23,25,27,33–35]. We found that fibrin gels cycled continuously at 8% strain show comparable increases in UTS as collagen gels (approximately threefold increase in gels stretched uniaxially at 10% strain for 8 days) [12] and even greater increases than reported in collagen-fibrin gels (~1.3-fold in gels stretched uniaxially at 10% strain for 8 days) [9]. Despite these large increases in tissue strength, the UTS of the fibrin gels (~30 kPa) is still substantially lower than those reported for collagen or collagen-fibrin gels (~60 kPa). As these gels are predominately composed of fibrin after only 9 days in culture [13], and fibrin is generally weaker than collagen [9], this outcome is to be expected. Alternatively, the gels in this study may have lower UTS values than previously reported due to minimal in-plane alignment compared with gels cultured while being uniaxially stretched and tested uniaxially [13].

4.2 UTS Increases Exponentially as a Function of Stretch Magnitude. In the present study, the UTS increased exponentially with increasing stretch, and this enhancement of tissue strength did not require continuous stimulation, exogenous growth factors, or extended culture duration—parameters previously thought to be required for a substantial increase in tissue strength [27,35,36]. It is unclear why notable increases in UTS require up to 5 weeks in other fibrin gel systems, although it may be due to passive decreases in thickness that may occur in the planar format relative to cylindrical geometries [35]. The substantial increase in UTS (approximately twofold) in intermitently stretched gels from only 8 days of mechanical conditioning observed in this study was consistent with our previous data [13]. The similarity in trends in UTS between the intermittent and continuous groups was, however, unexpected. The intermittently stretched gels had higher collagen density, and increased collagen density typically results in increased tissue strength [11,27].

4.3 Tissue Compaction is Both a Passive and an Active Response to Stretch. Compaction results in very large decreases in matrix volume (~80–95%), creates large changes in all intrinsic parameters, and can potentially obscure more meaningful changes resulting from cell-mediated remodeling. For example, stretch-induced trends in thickness and UTS are inverse of one another (see Figs. 2(a) and 2(b)), indicating that compaction, rather than true changes in fiber network strength, may dominate the observed increases in UTS. Furthermore, if one were to only examine the UTS of our gels as the only metric of tissue strength, it would appear that the introduction of a rest period does not impact stretch-induced matrix strengthening. The effect of introducing the rest period is only made apparent when investigating changes in failure tension, a remodeling metric independent of tissue compaction, as described in Sec. 4.4. In addition, we and others have shown that stretch-induced matrix compaction is not an entirely cell-mediated event. Acellular gels compact when stretched cyclically [9,13], albeit significantly less than cell-populated gels (approximately threefold less). These findings highlight the importance of measuring both intrinsic (compaction-dependent) and structural remodeling parameters to fully characterize changes in tissue properties due to mechanical conditioning.

4.4 Stretch-Induced Increases in Failure Tension are Contingent on a Rest Period. To investigate cell-mediated changes in tissue strength without the confounding effects of changes in tissue thickness due to compaction, researchers have attempted to minimize or eliminate matrix compaction during cyclic loading by allowing cells to fully compact gels prior to mechanical condition-

Journal of Biomechanical Engineering

MAY 2009, Vol. 131 / 051005-7
There are several limitations to the biaxial characterization measurements. During the biaxial testing, the fibrin gels rippled at the anchors at large bulk strains (above 10%) due to the low shear strength of the gels. Additionally, the “effective” elastic modulus was estimated from a single equibiaxial protocol following preconditioning assuming a Poisson ratio of 0.25. This value was determined in preliminary experiments that utilized four different nonequibiaxial stretch protocols (ε1/ε2=2:1, 1:2, and 1:1; data not shown). These protocols were run to allow for more complete constitutive modeling, which we have left for future studies. Finally, because the biaxial characterization device was developed after the continuous stretch experiments, only measurements of the intermittently stretched gels could be made.

4.6 Tissue Retraction is Dependent on Stretch Magnitude. To quantify both the ability of the cells to impart residual strains in the matrix through remodeling activities and the cells’ ability to actively contract the surrounding matrix, we utilized a retraction assay described previously [17]. Retraction, confusingly termed “stress-relaxation” or “release of tension” in previous studies utilizing collagen gels [30–32], occurs upon release of a compacted gel from the anchor and substrate. Whereas compaction occurs over a period of time and reflects cell-mediated remodeling of the gel [33,34], passive retraction occurs immediately upon the release of prestress generated within the matrix by cells during compaction [35]. Active retraction occurs on the order of minutes following the release and is due to the cells actively contracting within the matrix; this active phase is indicative of the phenotype of the cells, e.g., myofibroblasts contract the matrix to a greater extent than fibroblasts [18].

The passive retraction in both continuous and intermittently stretched gels was strikingly similar, demonstrating that the development of prestress across the matrix was dependent on the magnitude and not on the duration of stretch. In addition, it appears that there is a decrease relative to controls in passive retraction at 2% strain in both groups. This decrease in passive prestress is not accompanied by any change in tissue compaction or cell number relative to controls, and is clearly correlated with the level of stretch applied. In contrast to previous reports, no significant increase in active retraction with dynamic culture was observed at any stretch magnitude [8,39]. It has also been suggested that stretch-induced increases in cell contractility are due to the alignment of cells and collagen fibrils in the direction of tension, thus creating a summation of contraction. In our system, this alignment has been minimized by using equibiaxial strain, indicating that tissue alignment is not required for cells to impart forces across the matrix but may augment the active contractility of the cells [13].

4.7 Conclusions and Summary. In summary, in this study we systematically investigated how mechanical conditioning modifies cell-mediated remodeling of fibrin gels by measuring the structural and intrinsic parameters and active and passive metrics, each of which gives different but valuable insight. For example, cyclically loading a sample at low strain (~5%) for a few hours each day appears to be more beneficial in terms of increases in resistance to tensile stretch, collagen content, and cell number than continuous stimulation at higher magnitudes of strain. In addition, the methods employed in this work provide valuable tools for investigating tissue remodeling in planar tissues and also for the determination of key parameters in the culture of functional tissue substitutes. The findings from this study provide a step toward characterizing culture conditions for tissue equivalents, developing improved wound healing treatments, and understanding tissue responses to changes in mechanical environments during growth, repair, and disease states.

Acknowledgments

The authors would like to thank Dr. Glen Gaudette of Worcester Polytechnic Institute for the generous use of his extended phase image correlation software. We would also like to thank Dr. Jayson Wilbur for his assistance with statistical analysis, Dr. Marsha Rolle for her insightful commentary regarding the construction of the manuscript, and Angela Throm, Jeffery John, Christian Grove, Justine Roberts, Vanessa Lopez, Adriana Hera, Timothy Ebner, and Jacqelyn Youssef for their technical assistance. This work was supported in part by the U.S. Army Medical Research and Materiel Command (USAMRDC), Grant No. BFR008-1011-N00.

References


