Published in final edited form as:

J Mech Behav Biomed Mater. 2012 September ; 13: 25–35. doi:10.1016/j.jmbbm.2012.03.017.

Mechanics and kinematics of soft tissue under indentation are determined by the degree of initial collagen fiber alignment

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Abstract

While several studies have evaluated how the degree of collagen alignment affects the response of soft tissues to tensile loading, the role of fibrillar organization in indentation is less understood. Collagen-based tissue-equivalents (TEs) provide a convenient model system to explore structure-function relationships since their microstructural properties can be easily controlled during fabrication. The purpose of this study was to evaluate the role of initial collagen alignment on the mechanical and structural behavior of soft tissues subjected to indentation using TEs as a model system. Cell-compacted TEs with either isotropic or highly anisotropic fiber alignment were subjected to four-step incremental stress-relaxation indentation tests. The mechanical properties, collagen reorganization and 2D strain patterns were quantified at each indentation step and compared between groups. While no differences were seen in the peak force response, significant differences were seen in relaxation behavior, fiber kinematics and tissue strain. Specifically, highly aligned samples exhibited a slower relaxation rate, smaller changes in collagen fiber orientation, larger changes in strength of alignment, and larger strain magnitudes compared to isotropic samples. Results demonstrate the significant role that microstructural organization plays in mediating the response of soft tissues to a non-tensile (i.e., indentation) mechanical stimulus.

Keywords

indentation; collagen fiber alignment; mechanical response; soft tissue analog

1. INTRODUCTION

Soft connective tissues exhibit complex mechanical properties that depend on the composition and organization of individual tissue constituents and the interactions among the various components. The primary structural basis for many planar soft tissues is a network of Type I collagen fibers that imparts strength and stiffness under tensile load, while other non-collagenous proteins (i.e., proteoglycans, glycosaminoglycans, etc.) play a more important role in withstanding non-tensile loading. Unfortunately, many of the details regarding the specific roles of individual constituents in different functional environments remain unclear, as does how these constituents interact to provide unique and complex tissue properties.

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Towards understanding the interaction among constituents in more detail, our group and others have utilized collagen-based tissue-equivalents (TEs) as a simplified model system to evaluate properties and relationships of soft tissues (e.g., (Wood and Keech 1960; Grinnell and Lamke 1984; Guido and Tranquillo 1993; Eastwood et al., 1998; Roeder et al., 2002; Thomopoulos et al., 2005; Hu et al., 2009; Sander et al., 2009)). TEs are a particularly useful experimental system because organizational and compositional properties can be prescribed during formulation in controlled and predictable ways, which then facilitates the identification and measurement of isolated contributions of specific tissue components. For example, we recently used collagen-agarose co-gel TEs to evaluate the role of non-fibrillar matrix in tension and indentation, by varying sample *composition* (i.e., the quantity of non-fibrillar matrix) and then quantifying differences in response to mechanical load (Lake and Barocas 2011; Lake et al., 2011). In a similar way, the motivation of the current study is to investigate the role of tissue *organization* in mediating the response to a mechanical stimulus. In terms of evaluating the contribution of the collagen fiber network, TEs are attractive because different mold geometries can be utilized to vary fiber alignment. Specifically, collagen fiber alignment patterns that result from cell-mediated network compaction and reorganization have been predicted using computational models (Barocas and Tranquillo 1997; Nagel and Kelly 2011) and observed experimentally (Grinnell and Lamke 1984; Barocas et al., 1998; Eastwood et al., 1998; Costa et al., 2003; Wang et al., 2003; Kostyuk and Brown 2004; Thomopoulos et al., 2005; Jhun et al., 2009; Sander et al., 2011). Thus, TEs provide a model system within which the degree of anisotropy of the microstructural collagen network can be controlled and evaluated.

Several previous studies have explored the contribution of collagen fiber organization in *tensile* loading by evaluating the biaxial mechanical properties of cell-compacted gels of differing alignment (Thomopoulos et al., 2005; Thomopoulos et al., 2007; Jhun et al., 2009). While these studies elucidate some correlations between organizational and functional properties (e.g., more highly aligned samples exhibit higher modulus values), the exact nature of the relationship between structural and mechanical anisotropy remains unclear. For example, one study (Thomopoulos et al., 2007) concluded that the mechanical anisotropy of cell-compacted collagen gels in tension could not be explained by the degree of fiber alignment alone, which illustrates the complex nature of these relationships even in a simplified tissue analog system.

Many soft tissues, even those normally thought of as planar, experience complex *in vivo* loads that include shear and compression as well as tension (e.g., supraspinatus tendon (Flatow et al., 1994; Luo et al., 1998; Bey et al., 2002; Nakajima et al., 2004)). It is therefore imperative that we explore non-tensile loading configurations to supplement our existing knowledge of structure-function relationships in tension. Confined compression experiments of TEs (Knapp et al., 1997; Girtan et al., 2002; Chandran and Barocas 2004) have provided considerable insight, but are only one possible loading configuration, and were unable to evaluate the role of collagen organization because only isotropic samples were evaluated.

More recently, other studies have utilized large, macroscale indentation testing (as opposed to nanoindentation or atomic force microscopy) to characterize the anisotropic properties of soft tissues (Bischoff 2004; Cox et al., 2006; Cox et al., 2008a). Indentation testing is more relevant for certain types of tissues due to closer proximity to physiological loading. For example, fiber-reinforced tissues such as tendon and ligament can be subjected to macroscale indentation *in vivo* through interactions with neighboring anatomy near joints or when wrapping around bones. In these areas of indentation, the highly uniform collagen alignment typical of tendon/ligament becomes less organized, cell phenotype and gene expression is altered, and fibrocartilaginous tissue can develop as result (Koob and Vogel 1987; Giori et al., 1993; Rufai et al., 1996; de Palma et al., 2004). To date, however, it is not

clear how such differences in fiber alignment alter the behavior of these tissues in indentation, limiting our understanding of the normal adaptation to complex *in vivo* loading and the remodeling that may occur in injury and disease. More generally, it remains unknown what role the degree of initial (unloaded) collagen fiber alignment has in modulating the response of fiber-reinforced soft tissues to such loading environments. To this end, the purpose of this study was to evaluate the role of initial collagen organization on the mechanical and structural behavior of soft tissues subjected to indentation using cell-compacted TEs with prescribed initial alignment as a model system.

2. METHODS

2.1. Cell Culture

As described previously (Sander et al., 2011), neonatal human dermal fibroblasts (nHDFs, Clonetics, Walkersville, MD) were cultured in a 50:50 mix of Dulbecco's modified Eagles medium and Hams F12 medium (DMEM/F12, Invitrogen, Carlsbad, CA) with 10% fetal bovine serum (FBS, Hyclone Laboratories, Logan, UT), 100 units/mL penicillin (Invitrogen), 100 μ g/mL streptomycin (Invitrogen), and 2.5 μ g/mL amphotericin-B (Invitrogen). Cells were passaged at 100% confluency and harvested at passage 9.

2.2. Sample Preparation

Tissue-equivalent (TE) samples were prepared using a combined mold-grip system described previously (Sander et al., 2011). Two specific mold geometries were used in this study: rectangular molds in which the sample is secured uniaxially at two ends (1:0 arm-width ratio), and cruciform molds (1:1 arm-width ratio) in which the sample is secured biaxially at each of the four arms (Fig. 1A). Using similar molds, a previous study (Jhun et al., 2009) reported collagen fiber organization that transitioned from highly aligned to isotropic as the arm-width ratio of the molds decreased. For the current study, the 1:0 and 1:1 mold geometries were chosen to create samples at each end of alignment spectrum, referred to herein as "ANISO" and "ISO", respectively, because of the anisotropic and isotropic collagen fiber orientation they produce. Prior to casting, borosilicate rods, Teflon molds and Teflon pins (shown in Fig. 1A) were sonicated with detergent, rinsed in deionized water, and soaked in 70% isopropyl alcohol for 15 minutes. After air-drying, molds were soaked in 5% Pluronic F-127 (Sigma-Aldrich, St. Louis, MO) for 1 hour to prevent samples from adhering to the mold surfaces. In addition, 500 μ L of Pluronic was pipetted into the bottom of six-well plates and allowed to evaporate. Molds were then assembled with accompanying pins and rods and sealed within six-well plates using a thin layer of silicone-based high-vacuum grease (MSDS 01018817, Dow Corning, Midland, MI).

A 1.5 mg/mL collagen solution was prepared according to a previously described protocol (Sander et al., 2009; Raghupathy et al., 2011). The following were added sequentially (by volume fraction): 3% 1M 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES, Cellgro, Manassas, VA), 12.2% 0.1M NaOH (Sigma-Aldrich), 10% 10X Modified Eagle's Medium (MEM, Sigma-Aldrich), 6% FBS, 1% *L*-glutamine (Invitrogen), 0.1% penicillin-streptomycin, 0.1% amphotericin-B, and finally, 67.7% 2.2 mg/mL bovine dermal collagen (Organogenesis, Canton, MA). A pellet of centrifuged cells (nHDFs) was mixed with the collagen gel solution at 0.5×10^6 cells/mL. The collagen-cell mixture was pipetted into molds ($n=12$ each for ANISO and ISO molds) and kept at 37°C for 30 minutes to allow for collagen self assembly. After gel formation, samples were covered with high glucose DMEM (Invitrogen), supplemented with 10% FBS, 1% amphotericin-B, 1% penicillin-streptomycin, 0.1% insulin (Sigma-Aldrich), 50 μ g/mL ascorbic acid (Sigma-Aldrich) and 1 ng/mL TGF- β (R&D Systems, Minneapolis, MN). Four hours after the media was added, a sterilized straight dental pick was used to trace around the edges of each sample to prevent

adhesions between TEs and the molds or plastic. After incubation for 2 days (ANISO) or 3 days (ISO) to allow for cell-mediated compaction and reorganization of the collagen network, samples were washed three times in phosphate buffered saline (PBS, Cellgro, Manassas, VA) for 10 minutes each, then stored at 4°C until mechanical testing, which occurred within a few hours. Two samples from each group were not removed from the incubator, but instead were incubated for an additional 24 hours with sodium azide (NaN_3) to ensure no contribution from cells in these samples (Lijnen et al., 2001). Specifically, media supplemented with (by volume) 1% sodium azide (Sigma-Aldrich), 1% GM6001 matrix metalloproteinase inhibitor (Millipore, Billerica, MA), and 1% protease inhibitor cocktail (Sigma-Aldrich) was applied to the samples. After 24 hours, samples were washed three times for ten minutes in PBS and stored at 4°C until mechanical testing. The NaN_3 samples were each tested in the YZ orientation (described below) in order to compare mechanical parameters to non-treated samples.

Prior to mechanical testing, a custom parallel-blade cutting device was used to cut and remove two arms of the cross-shaped ISO samples, resulting in rectangular-shaped samples (Fig. 1A) with planar dimensions equal to those of the ANISO samples (~4mm wide $\times$ ~25mm long). Thus, samples of equivalent shape, but with varying initial fiber organization in the sample center, could be tested in indentation. Samples were briefly removed from PBS, and a fine speckling of Verhoeff's stain was applied to the top surface of the sample to enable image-based displacement tracking. As described in the following section, thickness was measured after placing each sample in the indentation device (for the YZ samples) or after mechanical testing was complete (for the XY samples). Average thickness values were 2.60 ± 0.06 mm and 1.73 ± 0.19 mm (mean $\pm$ 95% CI) for the ANISO and ISO samples, respectively. Finally, quantitative polarized light imaging (QPLI (Tower et al., 2002)) was used to capture alignment maps of each sample before indenting (Fig. 1B). QPLI computes angle and retardation values, corresponding to the average direction and strength of collagen fiber alignment, respectively. Small retardation values indicate in-plane isotropy while large values represent highly aligned, anisotropic organization. Using this measure, the initial microstructural organization resulting from cell-mediated compaction and reorganization was evaluated.

2.3. Mechanical Testing

Samples were placed in a PBS bath at room temperature and loaded in one of two custom-built test systems (Fig. 2) described previously (Lake et al., 2011). The full set of analyses could not be performed simultaneously for a single orientation of testing, therefore two orthogonal test setups and separate sample testing was required. In both test setups, the Y-axis was defined along the long axis of the sample, and two grips kept the y-length constant throughout testing. The X-axis was along the width of the sample, which was a free surface during testing. The Z-axis was through the thickness of the samples, and was the direction in which indentation was applied via a bar indenter. In the XY test setup (Fig. 2A), samples were positioned so that digital images could be captured in the sample's length-width plane in order to compute 2D strain and fiber alignment using QPLI. In the YZ test setup (Fig. 2B), samples were rotated 90° about the Y-axis, which allowed for visualization of the z-direction (sample thickness). In YZ tests, the undeformed sample thickness was computed after application of a tensile pre-stretch (described below) by evaluating static images in ImageJ (National Institutes of Health, Bethesda, MD), where the average length of manually-drawn lines across the sample thickness was calculated and converted to millimeters using the known width of the indenter as a calibration factor. Since the thickness of XY samples could only be measured post-testing (described below), displacement increments to be applied for the XY tests were computed based on average thickness values from the YZ tests. The loading protocol in both cases consisted of a uniaxial pre-strain of

5%, followed by four incremental stress-relaxation indentation steps of 10% of the sample thickness (up to 40% maximum indentation) at 10%/second, interspersed with 300-second relaxation intervals (Fig. 2C). Force values were captured at a sampling rate of 100Hz. In XY testing, QPLI image sets were captured after application of the *tensile* pre-strain (zero *indentation*) and immediately after each indentation step (10, 20, 30 and 40%). Individual images from these sets were also used for 2D strain tracking (described below). As in our previous study (Lake et al., 2011), force values were reported only for the YZ tests because XY forces exhibited some noise artifact resulting from physical elevation of the indentation platform. Importantly, the same loading protocol was used for both test configurations, so YZ forces were assumed to be representative of the mechanical response of samples in both XY and YZ testing. For each test configuration (XY and YZ) and sample geometry (ANISO and ISO), five samples were tested. After testing, the thickness of each XY sample was evaluated using a laser scan micrometer (Mitutoyo, Aurora, IL). Since thickness measurements at the center of the samples could be affected by artifacts from testing (e.g., any amount of permanent deformation), thickness was computed as the average of measurements obtained ~5mm on either side of the centerline. Thickness values for the ANISO and ISO XY samples were not statistically different from values measured for the YZ samples.

2.4. Collagen Assay

After mechanical testing, a small rectangular piece (4mm wide $\times$ 3mm long) was removed from the center portion of each sample by making two parallel cuts across the width (x-direction), and stored at -20°C . Collagen content was determined by measuring hydroxyproline as described previously (Stegemann and Stalder 1967; Neidert et al., 2002), except for the omission of lyophilization. Hydroxyproline values were converted to total collagen using 7.46 μg hydroxyproline per 1 μg of collagen (Dombi et al., 1993), and collagen concentration was calculated by dividing total collagen by sample dimensions (4mm $\times$ 3mm $\times$ thickness). In this case, collagen content was normalized by volume because of our ability to acquire a piece of each TE with very consistent dimensions and a high confidence in volume measures (note: *undeformed* sample-specific thickness was used for volume calculations).

2.5. Data Analysis

Peak and equilibrium force values were computed at each indentation step (10, 20, 30 and 40% of sample thickness) and grouped by sample type (ANISO or ISO). Relaxation behavior was evaluated by normalizing force data (by peak values) and by plotting group-averaged force curves vs. log-scale time.

Collagen fiber alignment was analyzed using QPLI. In order to quantify the relative anisotropy that resulted from cell-mediated compaction, fiber alignment maps of undeformed samples were evaluated. To track fiber kinematics due to indentation, QPLI image sets were also captured during mechanical testing. For the XY indentation samples, QPLI data (angle and retardation values) were averaged within each element of a 35×10 mesh (length $\times$ width; each element approximately $0.4\text{mm} \times 0.4\text{mm}$) imposed on the sample surface, at each indentation step (0, 10, 20, 30 and 40%). Element values from data acquired after the application of the 5% tensile pre-stretch but at 0% indentation (i.e., undeformed *indentation* configuration) were subtracted from each subsequent step (Fig. 4A, 5A), which were then averaged across all samples in the group ($n=5$ per group) to generate angle and retardation *difference* maps at each indentation step (Lake et al., 2011). Casting QPLI data onto a relatively coarse mesh allowed for the creation of difference maps while accounting for changes in sample geometry due to loading, and for the creation of group-averaged maps

while allowing for differences in geometry across samples within each group. In this way, the change in direction and strength of alignment due to indentation could be evaluated.

Two-dimensional strain in the XY plane was computed using digital image correlation (DIC) of images acquired at each indentation step (Fig. 6A). Images were 860 pixels $\times$ 640 pixels with approximately 0.045 mm/pixel. A 35 $\times$ 10 mesh was mapped onto the undeformed sample domain, and custom software based on the iterative Newton-Raphson method for sub-pixel displacements was used to compute the displacement field at each step (Pan et al., 2009; Raghupathy et al., 2011). Briefly, a fine mesh was created for each image set, and pixels corresponding to discrete nodal locations were tracked and used to compute a preliminary displacement field. The field was smoothed and interpolated at nodal locations to obtain an accurate estimate of the displacements and strains (refer to (Raghupathy et al., 2011) for more detail). Components of the 2D Green strain tensor (E) were computed relative to node coordinates in the undeformed mesh. Group-specific strain maps were generated by averaging the eigenvalues of the strain tensors for each element and by plotting contour-quiver plots of the principal strains for each group.

For all three types of map generated (alignment, retardation, and principal strain), a quantitative comparison of values was also performed. For each map, average and maximum parameter values across all elements were computed, and then evaluated with other samples in each group for statistical comparison. For the angle and retardation maps, since these values represent changes (+ or -) with respect to the undeformed state, the absolute value of these parameters was considered. First principal strain (E_{11}) values were always positive, so using the absolute value was unnecessary.

2.6. Statistical Analysis

Collagen concentration for the ANISO and ISO groups was compared using a two-tailed t -test. For the force data, unpaired t -tests were used to evaluate differences between peak forces at each indentation step, and at nine interpolated time points during stress-relaxation. Since taking the absolute value of the angle and retardation change data led to non-normally distributed data, these data were reported as median $\pm$ interquartile range, and non-parametric Mann-Whitney tests were used to assess differences in the average and maximum values between groups. T -tests were used to compare the average and maximum values of E_{11} at each indentation step. Finally, collagen concentration and peak force values for the samples treated with sodium azide were compared to untreated samples to identify any differences caused by contribution of cells. For all statistical analyses, Bonferroni corrections were used when multiple comparisons were performed, and statistical significance was defined as $p < 0.05$.

3. RESULTS

There were large differences in collagen fiber alignment for the undeformed samples due to differences in cell-mediated compaction for the 1:0 and 1:1 mold geometries (Fig. 1B). Retardation, which indicates the relative strength of alignment, was very low for the ISO samples, demonstrating a largely isotropic microstructural organization. Retardation for the ANISO group was significantly higher, indicating a much more strongly aligned network. Angle values for the undeformed samples also varied between groups (Fig. 1B): ANISO samples showed uniform alignment along the sample long axis, while the ISO samples exhibited alignment more evenly distributed in other directions, particularly near the center of the samples. Thus, the undeformed QPLI data verifies that cell-compacted TEs were fabricated with different initial collagen fiber organization; namely, nearly isotropic networks (i.e., ISO) and highly anisotropic (or nearly transversely isotropic) networks (i.e., ANISO).

The collagen concentration for the ANISO and ISO groups was 13.01 ± 1.21 mg/mL and 13.46 ± 1.69 mg/mL (mean $\pm$ 95%CI), respectively, which was not significantly different ($p=0.68$).

Peak force values were not different between groups at any of the load steps (Fig. 3A). Equilibrium force values (not shown) were approximately zero for all groups at all indentation steps, indicating full relaxation of indentation force that was consistent for all test conditions. In contrast, the time-dependent relaxation was different between groups (e.g., at 40% indentation step, Fig. 3B). Specifically, the ISO samples relaxed more quickly than the ANISO samples, with statistically significant differences ($p < 0.02$) apparent at the intermediate time-points.

For the parameter difference maps (Fig. 4B and 5B), gray elements represent no change relative to the undeformed configuration (e.g., $\Delta\theta=0$), while blue and red values correspond to negative and positive angle changes, respectively, with color intensity demonstrating magnitude of change. Angle difference maps (Fig. 4B) show increasing magnitude of change with increasing indentation depth, as fibers rotated away from the indenter (represented by dashed box). A comparison of the ANISO and ISO maps yields two apparent differences: the magnitude of change was much greater for ISO samples, while the spread of changes (i.e., breadth of affected tissue area) appears to be larger for the ANISO samples. Supporting the former observation, average and maximum (absolute value) angle change were significantly greater for the ISO than for the ANISO group at each indentation step (Table 1, Fig. 7A).

Retardation difference maps (Fig. 5B) also show increased magnitude of change with increasing indentation. However, in contrast to angle changes, the retardation increases for the ANISO group were much larger than for the ISO group, and the spread of affected area was also larger for the ANISO group. Evaluated quantitatively, average and maximum (absolute value) retardation change was significantly greater for the ANISO than for the ISO group at each loading step (Table 2, Fig. 7B). Importantly, the predominant retardation changes were negative, indicating a decrease in the strength of collagen fiber alignment as a result of the deformation, as was expected.

Two-dimensional 1st principal strain maps (Fig. 6B) show increasing strain at each indentation step, where the direction of strain was oriented parallel to the indenter width (i.e., samples expanded away from the long-axis). Throughout testing strains remained relatively small for the ISO samples (i.e., $<5\%$), but the ANISO samples exhibited very different strain maps, with maximum lateral strains of approximately 11% at the 40% indentation step. Average and maximum 1st principal strain values in the ANISO samples were significantly larger than in the ISO samples (Table 3, Fig. 7C).

In order to evaluate whether any lingering contribution from cells (after PBS washes) was significantly altering the response of TE samples under indentation, a subset of samples from each group ($n=2$) were cultured an additional day with sodium azide to disrupt cells (Lijnen et al., 2001). There were no significant differences between untreated and treated samples in terms of peak indentation force ($p > 0.32$) and collagen concentration ($p > 0.1$) for either group (note: NaN_3 data not shown).

4. DISCUSSION

In this study, the role of initial collagen fiber alignment on the mechanical and structural response to indentation was evaluated using tissue-equivalents (TEs). Samples with isotropic and highly anisotropic fiber alignment were successfully fabricated by taking advantage of direction-specific cues that different mold geometries (1:0 and 1:1 arm-width ratios)

imparted to cells as they reorganized the collagen fiber network during incubation. Using these microstructurally-varying TEs as a simple experimental model system, we found large differences in the behavior of isotropic and anisotropic soft connective tissues under indentation.

The most striking results were found in the 2D parameter maps (Figs. 4–6), which represent change in fiber alignment (orientation angle and retardation) and the magnitude and direction of principal strain. As demonstrated via strain maps, all samples were seen to expand laterally (in x-direction, Fig. 2) when loaded, particularly near the indenter, and the transverse strain decreased in magnitude as a function of distance from the indenter. Strain values were ~2–2.5 times larger in the ANISO samples compared to the ISO samples (Table 3, Fig. 6, Fig. 7C). For the ANISO samples, the fibers were initially aligned mostly along the sample long-axis (Fig. 1B), such that there was little structural resistance to expansion in the x-direction when indented. The ISO samples, on the other hand, had fibers initially oriented in all directions (Fig. 1B), including some in the lateral direction that resisted displacement in that direction. A recent computational study (Nagel and Kelly 2010) reported a similar result, where isotropic samples contained horizontally-oriented fibers that inhibited lateral expansion under unconfined compression. An earlier study (Cox et al., 2008a) using DIC to calculate strain from confocal images reported displacement patterns indicative of spreading away from the indenter (similar to the current study), but the lateral strains were similar in magnitude for both aligned and isotropic samples. Several differences in experimental protocol could explain the disagreement of this result with the current study (e.g., spherical vs. bar indenter, collagen gel vs. polyglycolic acid-fibrin scaffold, long vs. short incubation time).

Group-averaged angle and retardation difference maps also yielded differences between groups. At each indentation step, average $|\Delta\theta|$ values were ~2.5–3 times larger for ANISO compared to ISO samples (Table 1, Fig. 7A). With no preferred direction in the undeformed case (Fig. 1B), the deformed orientation angle of fibers in the center of the ISO samples changed dramatically (up to ~85° maximum angle change at 40% indentation) to accommodate and support the deformation applied by the indenter, demonstrating large deformation-induced realignment of the collagen network. In a computational study (Cox et al., 2006), fiber realignment was predicted $\pm 45^\circ$ from the predominant fiber direction when simulating indentation of a moderately aligned sample. The authors noted that fibers realigned in this way at large levels of indentation (>50%) in order to redistribute stresses over fiber directions away from the predominant fiber direction. Similarly, samples in the current study showed realignment of fibers towards directions $\pm 45^\circ$ from the predominant fiber direction (at 0°) and the indenter (at 90°), which was more pronounced for the ISO group than the ANISO group (Fig. 4). Another result that was observed, albeit less strongly, was an increase in the spread of affected area for ANISO samples. Thus, while ISO samples exhibited increased magnitude of $|\Delta\theta|$ values, the effect was more focused in the area near the indenter than it was in ANISO samples.

Compared to angle change data, the retardation difference maps exhibited a different trend, in which the magnitude of changes was much larger for the ANISO samples than for the ISO samples (Fig. 5). Specifically, average and maximum $|\Delta\delta|$ values were ~2.5–4 times larger for the ANISO group (Table 2). This result was not unexpected when considering the undeformed retardation values (Fig. 1B), where the ISO values were much smaller than the ANISO values. These values demonstrate a large difference in initial strength of alignment (isotropy vs. anisotropy), but also indicate how much decrease in retardation value was possible for either group (with 0° as the minimum δ value). Thus, the large initial retardation values for the ANISO group meant that a large drop in strength of alignment during loading was possible (from strongly aligned to moderately or poorly aligned). So, while orientation

angles did not change as dramatically for the ANISO group, these moderate angle changes corresponded with a broadened distribution of angle values such that the uniformity of the measured angle values (or alignment strength) decreased dramatically. Another interesting observation is that although the ANISO group showed large decreases in retardation, the ISO samples did not show a comparable increase in retardation due to alignment of the collagen; this may be due to the much lower lateral expansion in the ANISO samples, which may have limited the amount of collagen realignment.

In this study, peak force values were not different between groups (Fig. 3A), a result in striking contrast to the large differences between isotropic and anisotropic samples in *tensile* properties (Thomopoulos et al., 2005; Thomopoulos et al., 2007; Jhun et al., 2009). A computational analysis of soft tissue indentation also predicted similar forces up to ~30–35% indentation for isotropic, moderate and highly aligned TE samples (Cox et al., 2006). At higher indentation depths, the predicted force for the isotropic sample became larger due to a redistribution of stress onto orthogonal fibers (discussed earlier). It is unknown whether samples in the current study would show a similar behavior, since the maximum indentation in this study was 40% of sample thickness. In an experimental study by the same group (Cox et al., 2008b), indenter force for an isotropic sample was larger than for a highly anisotropic sample at lower levels of indentation (up to 25%); the authors acknowledged, however, that limited conclusions can be drawn from these results due to very low sample numbers. The similarity of peak force values for the two groups in the current study may be explained by two different mechanisms of supporting load. With high alignment in the y-direction (sample long-axis) of the ANISO samples, little resistance to load is provided laterally such that the sample strains significantly in the x-direction, but the aligned fibers seem to resist load in tight-rope-like manner. For the ISO samples, the poorly aligned network does not support load along the y-direction as effectively as the tight-rope-like structure in the ANISO samples, but instead has laterally-oriented fibers that resist indentation and limit expansion more effectively in the x-direction. In this way, planar TEs with dramatically different microstructural organization exhibited similar peak forces in indentation.

While peak force did not differ between groups, nor did total relaxation amount (~100% for both groups), the time-dependent relaxation behavior did vary with initial alignment (Fig. 3B). Differences in relaxation rate may be a thickness-effect since thicknesses differed by group: 2.60 ± 0.06 mm and 1.73 ± 0.19 mm for the ANISO and ISO samples, respectively. To investigate this possibility, a simple calculation was used to evaluate the potential contribution of thickness difference to relaxation rate in our experiments. Starting with an equation for fluid flow in a poroelastic material (Eqn. 8.5.13 of (Truskey et al., 2009)), a drainage time constant was approximated as:

$$t = \frac{\mu L^2}{kE} \quad (1)$$

where t is the characteristic drainage time, μ is permeability of water, L is path length of fluid flow, k is permeability, and E is modulus. With $L = (\text{sample thickness})/2$, k approximated using measured collagen content and an analytical solution for flow through an array of fibers (Sangani and Acrivos 1982; Stylianopoulos et al., 2008), and E approximated via tensile modulus measured experimentally (Evans and Barocas 2009), the computed drainage times were 6.7 seconds and 15 seconds for the ISO and ANISO groups, respectively. Interpolating average relaxation data (Fig. 3B) at these time increments yields loads that have relaxed to ~10% (ISO) and ~20% (ANISO) of the maximum force. Thus, thickness differences affect relaxation rates of these two groups (as evidenced by the drainage times), but other factors also likely play a role (as indicated by unequal normalized load values at group-specific drainage times). For example, faster relaxation for the ISO

samples compared to the ANISO samples may be due to differences in how relaxation occurs in each of the group-specific load-supporting mechanisms. Specifically, relaxation of load for the ANISO samples may occur by removing load off of fibers as they realign back towards the sample long axis, while ISO relaxation may occur through fluid exudation through the isotropic network. Finally, a contributing factor to differences in relaxation behavior could be an orientation-dependent difference in permeability for the two groups. Since results from the hydroxyproline assay suggest similar volume fraction for both groups, and our previous computational study (Stylianopoulos et al., 2008) calculated comparable permeability for flow across isotropic networks and for flow transverse to the preferred direction of anisotropic networks, the magnitude of this effect may be relatively minor compared to other contributing factors.

In order to control for possible cell-induced contributions to changes in properties, we evaluated a subset of samples from both groups that were incubated with sodium azide (NaN_3) for 24 hours prior to testing. Lack of differences between NaN_3 samples and non-treated samples (in terms of peak force and collagen concentration) suggests that there was at most a minor contribution from cells in the non-treated group.

The structure-function relationships of TEs elucidated in this study may also be applicable for understanding native soft tissues, but an important limitation in this regard should be noted. While TE samples and many connective tissues share Type I collagen as their primary load-bearing structural protein, the manner in which the collagen network is organized in TEs does not replicate the multilayer nature of some native tissues. Previous studies using scanning electron and confocal microscopy have shown a loose arrangement of collagen fibrils with visible banding typical of native collagen in acellular gels and densely arranged collagen fibrils in cell-compacted tissue equivalents (Roeder et al., 2002; Voytik-Harbin et al., 2003; Lee et al., 2008; Helary et al., 2010; Lake and Barocas 2011; Sander et al., 2011). Although such organization is simpler than the hierarchical, multilevel organization of some soft tissues (e.g., tendon and ligament), the motivating aim of using TEs is not to recapitulate any specific native tissue, but instead to create a simplified analog using the principal constituents of native tissues in order to explore general properties of materials constructed from such components. Nevertheless, one should keep in mind potential differences in mesoscale structural organization when extrapolating properties of TEs to a specific tissue of interest.

In conclusion, this study utilized a simplified tissue-analog model system to evaluate the role of initial collagen fiber alignment on the mechanical and structural behavior of soft tissues loaded in indentation. While no differences were seen in the peak force response of nearly isotropic (ISO) and transversely isotropic (ANISO) samples, significant differences were seen in relaxation behavior and in both fiber- and tissue-level kinematics, demonstrating the significant role that microstructural organization plays in mediating a tissue's response to a non-tensile mechanical stimulus. These results are also instructive in considering how the degree of fibrillar organization affects how soft connective tissues respond *in vivo* to complex loads. For example, within areas of tissue that are subjected to compressive loading, reorganization of the collagen fiber network (from high to low anisotropy) may allow the tissue to minimize lateral strain, modulate fiber kinematics, and/or alter rate of relaxation in order to meet the functional demands of the loading environment. Of course, during normal adaptation to physiological loading as well as remodeling that occurs in injury and disease, both the *organization* of the collagen network and the *composition* of the non-fibrillar matrix are altered. The current study, together with results of our previous compositionally-varying TE study (Lake et al., 2011), demonstrates how changes in the microstructural properties (i.e., organizational and compositional) of soft tissues lead to dramatic differences in the macroscale mechanical response to indentation. In

addition to elucidating properties and behavior of native tissues, such detail on structure-function relationships can also inform tissue engineering approaches on how to create constructs of appropriate compositional and organizational properties to be able to provide necessary function within a complex *in vivo* mechanical environment.

Acknowledgments

The authors thank Sandy Johnson for culturing cells and for performing the hydroxyproline assay. We also gratefully acknowledge the financial support of the National Institutes of Health (R01-EB005813 and F32-EB012352)

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Fig. 1.

(A) Photographs demonstrate mold-grip system and sample appearance after incubation for ANISO and ISO groups, with dashed lines indicating location of cuts that were made to trim ISO samples to rectangular shape; (B) alignment maps of representative samples prior to indentation show much stronger alignment for ANISO samples than ISO since orientation of the red lines of alignment maps indicates direction of collagen alignment, and the length of lines and the underlying gray scale retardation values indicate strength of alignment (black area in middle of sample is location of indenter)

Fig. 2.

Schematics and photographs show orthogonal indentation setups for (A) XY and (B) YZ testing; in both cases, the sample was held fixed in Y-direction and indenter was displaced in the Z-direction; (C) displacement-time plot illustrates the four-step incremental stress-relaxation indentation test protocol used in this study (10% indentation step followed by 300 second relaxation)

Fig. 3.

(A) While peak forces increased with indentation, there were no differences between values for the two groups; (B) significant differences were seen in relaxation behavior (shown at 40% indentation step), where the ISO samples relaxed more quickly than ANISO in the intermediate time period (n=5 per group; mean $\pm$ 95%CI; * for $p<0.05$)

Fig. 4.

(A) Angle difference maps were created by computing the change in angle values between the undeformed state and each indentation step for the elements of a 2D mesh and plotting group-averaged $\Delta\theta$ (example shown for a ISO sample at the 40% indentation step); (B) both groups show increased magnitude of change with increasing indentation (represented by increased color intensity), however ISO show larger magnitude of changes overall and ANISO maps indicate a larger spread of affected area compared to ISO maps (gray values = no change, red = + change, blue = - change; n=5 per group)

Fig. 5.

(A) Retardation difference maps were created by computing the change in retardation values between the undeformed state and each indentation step for the elements of a 2D mesh and plotting group-averaged $\Delta\delta$; (B) while the ANISO maps show large changes in retardation that increase in magnitude and spread with increasing indentation, the ISO maps show very small changes that are quite consistent for maps at all indentation steps (gray values = no change, red = + change, blue = - change; n=5 per group)

Fig. 6.

(A) Digital image correlation was used to analyze the change in texture between an undeformed image and images captured at each indentation step to compute 2D strain for each sample; (B) group-averaged 1st principal strain values plotted on a representative mesh show strains oriented parallel to the indenter that increased with increasing indentation, where the magnitude of strain values was much larger for the ANISO samples than for the ISO (n=5 per group)

Fig. 7.

Quantitative comparison of group-specific 2D parameter maps (shown in Figs. 4–6) yields statistically significant differences between average values of (A) absolute value angle change (median $\pm$ interquartile range), (B) absolute value retardation change (median $\pm$ interquartile range), and (C) 1st principal strain (mean $\pm$ 95% CI); ISO exhibited larger angle changes but smaller values of retardation change and strain (shown for 40% indentation step; n=5 per group; * for p<0.05)

Table 1

Values of average and maximum (absolute) angle change for elements of angle difference maps

	ANISO						ISO					
	$/\Delta\theta$ avg			$/\Delta\theta$ max			$/\Delta\theta$ avg			$/\Delta\theta$ max		
	25 th	50 th	75 th	25 th	50 th	75 th	25 th	50 th	75 th	25 th	50 th	75 th
Percentile												
10%	1.24	1.63	1.67	8.75	11.30	13.33	2.50	3.59	5.23	38.80	65.04	75.63
20%	1.24	1.54	2.04	6.49	10.81	16.54	3.00	4.42	6.77	62.02	78.92	87.60
30%	1.85	2.25	2.74	11.48	13.81	23.30	4.97	5.30	8.59	73.55	83.60	87.42
40%	2.34	2.61	3.82	20.34	24.29	29.32	6.16	6.42	9.81	79.29	87.42	88.37

median $\pm$ interquartile range; n=5 per group; all comparisons between ANISO and ISO were significantly different ($p<0.05$)

Table 2
Values of average and maximum (absolute) retardation change for elements of retardation difference maps

	ANISO						ISO					
	<i>/Δδ avg</i>			<i>/Δδ max</i>			<i>/Δδ avg</i>			<i>/Δδ max</i>		
	25 th	50 th	75 th	25 th	50 th	75 th	25 th	50 th	75 th	25 th	50 th	75 th
Percentile												
10%	2.74	3.46	3.75	16.33	18.91	22.75	1.00	1.18	1.45	6.72	8.87	9.56
20%	3.73	4.68	4.81	21.62	22.91	26.34	1.05	1.28	1.53	7.31	10.54	11.09
30%	5.14	5.66	6.57	29.49	30.88	34.78	1.46	1.77	2.03	10.23	11.94	16.59
40%	6.61	7.51	8.40	36.79	40.26	45.79	1.61	1.79	2.37	11.33	14.21	18.04

median ± interquartile range; n=5 per group; all comparisons between ANISO and ISO were significantly different (*p*<0.05)

Table 3

Values of average and maximum 1st principal strain for elements of 2D strain maps

	ANISO		ISO	
	E_{11} avg	E_{11} max	E_{11} avg	E_{11} max
10%	0.014 ± 0.003 *	0.041 ± 0.009 *	0.007 ± 0.003 *	0.019 ± 0.007 *
20%	0.023 ± 0.007 *	0.061 ± 0.018 *	0.009 ± 0.003 *	0.025 ± 0.008 *
30%	0.030 ± 0.009 #	0.082 ± 0.025 #	0.014 ± 0.006 #	0.038 ± 0.014 #
40%	0.042 ± 0.014 *	0.113 ± 0.037 *	0.017 ± 0.006 *	0.049 ± 0.016 *

mean ± 95%CI; n=5 per group; for comparisons between ANISO and ISO,

* indicates $p < 0.05$ and

indicates $0.05 < p < 0.1$