Left ventricular perimysial collagen fibers uncoil rather than stretch during diastolic filling

Abstract The collagen fibers in the myocardium are initially wavy, suggesting that they may not be directly stretched for a portion of diastolic filling. To test whether the fibers gradually straighten and at what left ventricular (LV) pressure they become straight, 24 isolated, arrested rat hearts were fixed at physiologic diastolic LV pressures and changes in collagen structure were examined. As LV pressure increased, mean (±SE) sarcomere length increased (1.80 ± 0.02 to 1.88 ± 0.02 from 0 mmHg to 26.3 ± 4.1 mmHg) while the tortuosity of the perimysial fibers (fiber length/midline length) decreased (1.088 ± 0.014 to 1.031 ± 0.006 from 0 mmHg to 26.3 ± 4.1 mmHg). Transmural variations in collagen structure paralleled the trends in sarcomere length (epicardial regions had longer sarcomeres and straighter collagen fibers than endocardial regions). These results indicate that there is a tight coupling between perimysial collagen fibers and myocytes, consistent with the nonlinear pressure-volume and pressure-sarcomere length relationships.

Key words Cardiac mechanics – extracellular matrix – picrosirius red – myocyte morphology – tortuosity

Introduction

The resting myocardium, like most soft biological tissues, has a nonlinear stress-strain behavior. This nonlinear behavior has partially been attributed to the properties of the collagen extracellular matrix (25), which comprises only 2–5% of the tissue by weight (29). In biomechanical tests on ligaments and tendons (30), it has been shown that the uniaxial behavior of collagen is linear at strains between ~2 and ~8% with a tensile stiffness (tangent elastic modulus) ranging from 500–1200 MPa. This stiffness is much greater than that observed in passive myocardium at even higher tensile strains (100–500 KPa at 10–15% strain (23)). This discrepancy is too great to be explained by the small volume fraction of collagen in the myocardium: if all the myocardial stiffness were attributed to fibrillar collagen tension and the cardiac collagen properties were similar to those of ligament or tendon, one would expect an elastic modulus of 30–50 MPa for myocardium. Another important difference between the properties of the uniaxial tendon and the myocardium is the nonlinearity. The tensile behavior of a tendon is linear at strains between ~2 and ~8%, while the myocardium has nonlinear properties over a large range of strains. It is well established from the biomechanical studies of ligaments and other tissues (4, 24) that the collagen fibers are “crimped” or wavy in the unloaded configuration and their unbending is related to the nonlinear stress-strain behavior. This crimp may be related to the helical nature of the primary structure of the amino acids, i.e., the repeated sequence of glycine-X-Y. Furthermore, wavy collagen structures appear in many tissues from blood vessels...
to intestine to skin (1, 12, 22, 24). In tissues that are more nonlinear (e.g., skin), the collagen fibers are more coiled than the planar crimp apparent in tendon. The coiled nature of those tendon-like structures may be associated with the large deformations and nonlinearity of their stress-strain behaviors.

Caulfield and Borg (2) examined collagen structure in the normal left ventricular myocardium by scanning electron microscopy, which revealed a complex hierarchy including small endomysial fibers connecting myocytes together laterally and endomysial and perimysial weaves that envelop individual cells and group sets of cells together. They also described “tendons” passing through the tissue that appeared coiled. Robinson and coworkers (26) noted that in papillary muscles these larger collagen fibers are coiled in the unloaded state and tended to straighten with increasing passive load. The coiled perimysial fibers in the papillary muscle were still not completely straight in the loaded configuration. Factor and coworkers (6) studied rat hearts fixed at high ventricular pressures and found that the collagen fibers appeared to uncoil at high pressures. The disappearance of the crimp pattern in tendons corresponds to the transition to linear elastic range which occurs at relatively low strains (0.5 – 2 %) (4). Therefore, since the nonlinear stress-strain behavior of the passive myocardium continues to greater strains (10 – 15 %), it is possible that the myocardial collagen fibers do not become completely straight over the normal range of diastolic filling pressures.

Hence, the objective of this study was to measure changes in the structure of perimysial collagen fibers in the normal myocardium in the physiologic range of diastolic ventricular pressures and determine whether they stretch or merely straighten as a function of left ventricular load. As the ventricle was distended and the tissue stiffened, the perimysial fibers became less coiled and the straightening tended to reach a limit in parallel with lengthening of the sarcomeres. This suggests that sarcomere stretch may be limited by the connective tissue (7). Moreover, the collagen fibers appear to gradually uncoil but not stretch during normal diastolic filling which is consistent with the observation that the low area fraction alone cannot account for the high compliance of the passive myocardium. The compliance may be due to another protein such as titin (15) or the mechanical behavior associated with straightening of these wavy fibers (17).

### Methods

#### Experimental protocol

Twenty-four Sprague-Dawley rats were anesthetized with sodium pentobarbital (100 mg/kg), ventilated with air, and the heart exposed via median sternotomy. All studies were performed according to AALAC guidelines for the use of animals in research; protocols were approved by the UCSD Animal Subjects Committee. The aorta was clamped and the heart arrested via an apical injection of 1 – 2 ml of hypothermic hyperkalemic arrest solution. The arrest solution contained (in g/l): NaCl 6.90; NaHCO₃ 2.29; KCl 4.48; Glucose 2.0. 10,000 u/l of heparin and 30 mM 2,3 butanedione monoxime (BDM) were added to the solution to prevent clotting and myocyte contracture, respectively. The heart was rapidly excised and weighed. The coronary vessels and myocardium were cleared of blood via perfusion with an aortic cannula. A vent (polyethylene tubing) was inserted into the LV through an apical stab wound to provide drainage of residual fluid in the LV. A small balloon was inserted into the LV through the mitral valve and secured with a purse string suture. The balloon was attached to a volume infusion pump and pressure transducer. The pressure transducer was zeroed at the level of the heart. Fluid was infused into the balloon at a rate of ~1 ml/min to a maximum pressure of 25 mmHg and then withdrawn to the original volume. The heart was preconditioned with 2 – 5 cycles until the behavior was repeatable (±0.005 ml at the same final pressure) (23). All data were acquired within 20 min after arrest.

To examine structural changes over the entire diastolic range of LV pressures, the hearts were randomly placed into one of four groups (n = 6 each) with two groups at relatively low stiffnesses (zero pressure (~0 mmHg) and low pressure (~5 mmHg)), one group near the transition from low to high stiffness (medium pressure (~10 mmHg)), and finally, a high stiffness group (high pressure (15 – 40 mmHg)). The balloon was inflated to a volume that approximated the desired LV pressure, and the heart was perfused with 2.5 % buffered-glutaraldehyde via the aortic cannula. Perfusion was stopped after the ventricle was uniformly fixed at the distending volume (by visual inspection of tissue color and palpation of tissue ~ 5 – 15 minutes). Pressure in the LV was monitored during fixation and if it increased ~5 mmHg above the desired level, flow was stopped, the ventricle allowed to drain through the vent and the cannula repositioned to prevent further aortic insufficiency. The balloon was then deflated and removed from the ventricle and an equatorial ~2 mm thick ring was cut from the mid-LV for histologic and geometric measurements. Outer and inner diameter were measured within ±0.25 mm at four locations around the
LV circumference. The ring was then stored in buffered-glutaraldehyde for 1 – 5 days.

Histologic preparation and measurements

The equatorial ring was embedded in paraffin and 10-μm sections cut parallel to the epicardium in the lateral LV freewall at 200-μm intervals through the wall thickness. As the wall thinned with increased ventricular volume, the number of sections obtained from each heart decreased. The unloaded hearts (zero pressure group) typically yielded 10 – 11 sections, while in the high pressure hearts, there were eight to nine sections. Six sections were selected from each heart: two outermost (epicardial), two central (midwall), and two innermost (endocardial). The sections were cleared of paraffin, brought to water through a series of graded ethanols, and stained for 90 min in picrosirius red (0.1 % w/v sirius red F3BA in saturated picric acid) to enhance the birefringence of the fibrillar collagen. For all subsequent histologic procedures, the observer was blinded to the source of the tissue.

Sarcomere length was measured to quantify local myocyte stretching associated with ventricular distention. Picric acid in the stain also enhances the birefringence of the sarcomeres, so it was possible to measure sarcomere length in these paraffin embedded sections. The section was rotated on a circular stage of the microscope (Nikon Optiphot-2) under polarized light (100× oil immersion objective) until the A-bands of the sarcomeres had the greatest intensity (45° between the polarizing axes). The image was digitally acquired (Sony CCD/RGB camera and Apple Macintosh Quadra 900 host with DT-2255 frame grabber) and the length of 10 adjacent sarcomeres measured for 10 sets in each slide using image analysis software (NIH Image 1.47). The system was calibrated with an objective micrometer yielding a resolution of 8.05 pixels/μm (~0.6 % accuracy). To assess shrinkage associated with paraffin embedding (14), 10-μm cryosections were made from separate equatorial slices in three of the hearts (two at zero and one at high pressure). The tissue was stained with the techniques of Canham et al. (1) using a similar process as described above except rehydration started at 70 % ethanol and picrosirius red staining occurred for only 30 min.

The waviness of the perimysial collagen fibers was quantified by the “tortuosity” of the path they follow. The sections were rotated on the circular stage of the microscope (40× objective) under polarized light until the collagen fiber was best illuminated (~30 – 60° between the polarizers). Because the goal was to best illuminate the collagen fibers along their length, and not to extinguish the muscle, the A-bands of the sarcomeres were still partially illuminated and appeared green. Therefore, the red channel from the color camera was digitally acquired because it revealed the best contrast between the collagen fibers (red-yellow) and the background muscle (green). The path of the collagen fiber and its midline period length were traced with the mouse using line segments sufficiently short that the fiber appeared smooth. Tortuosity was defined as the ratio of total length to midline length. Ten random fibers (each spanning at least 50 μm) were measured near the center of the tissue in each section.

An optically anisotropic object has four extinction angles 90° apart and is maximally bright at the four intermediate positions. This property was used to quantify the regional orientation of collagen in these samples. The area fraction of birefringent collagen was measured at two orientations, 45° apart, one corresponding to the extinction angle of the muscle (0 or 90°) (Darkmuscle) and one corresponding to the maximum intensity of the A-band birefringence (45°) (Bright-muscle). In this manner, the portions of the collagen fibers aligned parallel or perpendicular to the muscle were extinguished in the dark-muscle view and illuminated in the bright-muscle view while those aligned near 45° to the muscle axis were illuminated in the dark-muscle view and extinct in the bright-muscle view. The total area of birefringent collagen is a union of the area illuminated in each orientation, not simply a sum of the two.

To measure the local orientation of the collagen fibers, the tissue section was rotated on the circular stage of the microscope (40× objective) under polarized light until the muscle was extinguished, and a 24-bit color image acquired. The RGB color image was transformed into three eight-bit black-and-white images corresponding to hue (dominant wavelength), saturation (color purity) and brightness (image intensity) (17). The area of birefringent collagen was obtained by thresholding the brightness channel of the polarized image. The corresponding colors of the birefringent collagen were obtained from the hue channel of this same image. The polarizing and analyzing filters (not the tissue sample) were then rotated 45° to create the bright-muscle view that superimposed exactly with the dark-muscle view. The images were analyzed similarly, but the threshold criterion was relaxed to include some of the birefringent muscle. The collagen was then separated from the muscle by manual identification and extraction. The colors in the final images were used as an aid for identification because most collagen fibers appeared red-yellow. The brightfield view was acquired and the blue channel of the RGB image thresholded to determine area of tissue (excluding extracellular space, vessel lumen, and sectioning artifact). The area fraction of birefringent collagen at each orientation was calculated as the area of birefringent collagen/area of tissue.
Fig. 1 Effect of changing the orientation of the polarizers on the acquired images. Circles on right-hand side show the orientation of the polarizing filters. A) Birefringent collagen when the polarizers are aligned parallel to the muscle fiber axis (dark-muscle). Fibers are illuminated when they are oriented near 45° from the muscle axis. B) Polarizers rotated 45° from muscle fiber axis (bright-muscle). Collagen aligned parallel to the muscle axis is illuminated. C) Superposition of images in A and B. All orientations of fibrillar collagen are acquired.

The bright- and dark-muscle images were superimposed to calculate the total area fraction. Figure 1 demonstrates how the data acquired from the bright and dark muscle images can be combined to form a continuous fiber. The proportion of birefringent collagen observed at each orientation was calculated as the area in the bright-muscle orientation (or dark-muscle)/total area. Similarly, the two images were joined through a logical “and” operation to find the pixels that were illuminated in both views which constituted the overlap between the two orientations.

Laser scanning confocal microscopy

Dolber and Spach (5) have recently shown that picrosirius red fluoresces under either conventional fluorescent or laser scanning confocal microscopy. Therefore, we visualized a single picrosirius red stained collagen fiber from an unloaded heart using this technique. Using a Leica laser scanning confocal microscope with a 63× oil immersion objective, 28 0.3-μm thick optical sections were obtained for a single collagen fiber using both fluorescein and rhodamine standard filter sets. In these images, the collagen fiber was only illuminated by the rhodamine filter set. However, there was significant background autofluorescence of the muscle in both images. Therefore to extract the data for the fluorescent collagen fiber alone, the pinhole size of the laser was adjusted for the fluorescein image until the background muscle intensity matched that in the rhodamine image. The pixel values of the fluorescein image were then digitally subtracted from those in the rhodamine image. The 3D geometry of the collagen fiber was then reconstructed from the 28 images using NIH Image 1.47 on a Macintosh computer (Quadra 900). The geometry could be viewed either by rotating the collagen fiber around an arbitrary axis or by calculating vertical sections through the sets of images.

Statistical analysis

Linear regression analysis was used to test the significance of overall trends in the data. Typically, data were averaged for each heart to provide the proper degrees of freedom for the analyses. To evaluate specific differences between the pressure groups, a one-way analysis of variance (ANOVA) and post-hoc Fisher’s Protected Least Square Differences (PLSD) comparisons were performed on mean data where the pressure group (zero, low, medium, and high) was used as the independent factor. Finally, to examine the effects of depth on the histologic measurements, a two-way ANOVA and the same post-hoc PLSD analysis were used where both pressure and depth (epicardial, midwall, and endocardial) were independent factors.

Results

Heart and body weights were not different for the animals in the different pressure groups (342 ± 30 g and 1.16 ± 0.09 g, respectively for the pooled data). The isolated arrested rat left ventricle exhibited a repeatable nonlinear pressure-volume curve as observed previously (16, 23). The curves were similar for all hearts; in particular, the LV volume at 10 mmHg was not statistically different between the groups (0.224 ± 0.071 ml for the pooled data, p > 0.10). Left ventricular volume and outer and inner diameters increased while wall thickness decreased with increasing LV pressure (Table 1). The LV volume of the zero pressure group represents the equilibrium volume for those six hearts, which again was comparable to the entire group.

Alterations in microstructure with pressure

Sarcomere lengths increased with LV pressure (p < 0.0001 using regression) as shown in Table 2. Two-way ANOVA on data averaged for each location in each heart revealed significant effects both of pressure (p < 0.0001) and depth (p < 0.05) with no significant interaction (p = 0.56). The sarcomere lengths measured in the cryosections were
Table 1 Geometric and experimental parameters (mean ± SE) at time of fixation in the 24 isolated arrested rat hearts included in this study. Volume was infused into the balloon to approximate the desired pressure and the tissue was fixed with buffered glutaraldehyde. Ventricular geometry was measured from an equatorial ring. All statistics were performed with a one-way analysis of variance.

<table>
<thead>
<tr>
<th>Group</th>
<th>Pressure (mmHg)</th>
<th>Volume* (ml)</th>
<th>Outer Diameter (mm)</th>
<th>Inner Diameter (mm)</th>
<th>Wall Thickness (mm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Zero</td>
<td>0.9±0.4</td>
<td>0.21±0.003</td>
<td>11.6±0.2</td>
<td>4.6±0.2</td>
<td>3.5±0.1</td>
</tr>
<tr>
<td>Low</td>
<td>5.9±0.5</td>
<td>0.34±0.021</td>
<td>12.0±0.2</td>
<td>6.0±0.2</td>
<td>3.0±0.1</td>
</tr>
<tr>
<td>Med</td>
<td>10.8±0.4</td>
<td>0.378±0.017</td>
<td>11.8±0.2</td>
<td>6.3±0.3</td>
<td>2.8±0.1</td>
</tr>
<tr>
<td>High</td>
<td>26.3±4.1</td>
<td>0.531±0.025</td>
<td>12.6±0.2</td>
<td>7.5±0.3</td>
<td>2.6±0.1</td>
</tr>
</tbody>
</table>

p-value <0.0001 <0.0001 <0.02 <0.0001 <0.0001

* Includes 0.09 ml volume of unloaded balloon and cannula.

7 ± 4 % longer than those measured at similar depths in adjacent paraffin-embedded rings. The sarcomere lengths measured in the unloaded and high pressure hearts indicated similar amounts of paraffin induced shrinkage for both groups (6.5 % and 8 %, respectively).

Increased LV pressure had a noticable effect on the appearance of the perimysial collagen fibers. Figures 2 and 3 show tissue stained with picrosirius red from hearts fixed at zero and high LV pressure, respectively. In the unloaded tissue, the fibers appeared coiled and wavy, while they appeared straightened in the tissue fixed at high pressure. Consequently, the tortuosity (±SE) of the collagen fibers decreased from 1.088 ± 0.009 to 1.031 ± 0.005 with increasing ventricular load from 0.9 ± 0.4 to 26.3 ± 4.1 mmHg indicating straightening or uncoiling of the collagen fibers (p < 0.0001 using regression) (Fig. 4). Tortuosity tended to plateau, reaching a limit of 1.02 – 1.05 near 15 – 20 mmHg.

Rotating the axes of polarization allows quantification of the local orientation of collagen fibers and is more

Table 2 Summary of effect of ventricular load on histologically measured parameters (mean±SE). All depths were averaged for each heart to calculate the mean and standard error. With increased ventricular pressure, myocyte sarcomere length increased as tortuosity of the collagen fibers decreased indicating that the collagen fibers were uncoiling or straightening. Also indicative of straightening, the proportion of collagen in the bright-muscle view increased and that in the dark-muscle view decreased. Finally, independent of the coiled structure, total and bright collagen area fraction increased with load. Statistics performed with a two-way analysis of variance.

<table>
<thead>
<tr>
<th>Group</th>
<th>SL (μm)</th>
<th>Tortuosity</th>
<th>Proportion of collagen (%)</th>
<th>Bright</th>
<th>Area fraction (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Bright</td>
<td>Dark</td>
<td>Overlap</td>
</tr>
<tr>
<td>Zero</td>
<td>1.795±0.008</td>
<td>1.088±0.009</td>
<td>73.8±1.4</td>
<td>46.9±1.1</td>
<td>16.9±0.5</td>
</tr>
<tr>
<td>Low</td>
<td>1.834±0.007</td>
<td>1.081±0.011</td>
<td>77.3±1.2</td>
<td>42.3±1.9</td>
<td>15.7±0.9</td>
</tr>
<tr>
<td>Med</td>
<td>1.842±0.016</td>
<td>1.051±0.012</td>
<td>80.6±1.4</td>
<td>32.9±2.3</td>
<td>10.1±0.9</td>
</tr>
<tr>
<td>High</td>
<td>1.882±0.040</td>
<td>1.031±0.005</td>
<td>83.9±1.9</td>
<td>34.7±4.0</td>
<td>14.5±1.9</td>
</tr>
</tbody>
</table>

p-value <0.0001 <0.0001 <0.0001 <0.0001 <0.0001 <0.0001 <0.0005 <0.0001
objective than digitizing the tortuous path. In the unloaded configuration, the coiled collagen fibers were illuminated relatively evenly in the bright- and dark-muscle views with similar sized regions of light and dark (Fig. 2 – see arrows). In contrast, when the heart was fixed at high ventricular load, the straightened fiber was illuminated almost completely in the bright-muscle view and virtually extinct in the dark-muscle view (Fig. 3 – see arrows).

The proportion of birefringent collagen observed in the bright-muscle view (%Bright) increased with LV pressure indicating straightening of the fibers (p < 0.0001 using regression). Post-hoc analysis from a two-way ANOVA showed %Bright both in the high- and medium-pressure groups was significantly greater than in either the low- or zero-pressure groups. Similarly, the proportion of birefringent collagen that was observed in the dark-muscle view (%Dark; i.e., oblique to the muscle fiber axis) decreased with increasing LV pressure (Table 2) (p < 0.0001 using regression), also indicating straightening of the collagen fibers. A two-way ANOVA revealed differences due to pressure (p < 0.0001) identical to those for the bright-muscle view, except that the means decreased as the pressure increased. The amount of overlap (%Overlap) between the two orientations also decreased as LV pressure increased (Table 2).

Regression of parameters independent of load

To verify whether the tortuosity and proportion of birefringent collagen measurements both reflected unwinding of coiled fibers, the proportions of birefringent collagen were plotted against the tortuosity measurements for each individual section (Figure 5). As the collagen fibers straightened, the local orientation of the fibers should become more aligned with their major fiber axes and hence parallel to the myocytes, suggesting that the %Bright should increase and %Dark should decrease as the fibers straightened. Linear regression revealed statistically significant correlations between all three orientation measurements (%Bright, %Dark, and %Overlap) and tortuosity (p < 0.0001; p < 0.002; and p < 0.0001, respectively), consistent with this explanation, suggesting that both techniques measure the same uncoiling phenomenon.

Finally, to evaluate the direct interaction between straightening of the collagen fibers and stretching of the

Fig. 3 Picrosirius red-polarization photomicrograph from a heart fixed under high load for the bright (A) and dark (B) muscle views. When compared with Fig. 2, the collagen fibers are straighter. Furthermore, they are uniformly illuminated in the bright-muscle view and virtually extinct in the dark-muscle view (See arrows). (40X objective).

Fig. 4 Collagen fiber tortuosity as a function of pressure. Each point represents the mean for all blinded measurements (~60) for each heart. As LV pressure increased, the collagen fibers uncoiled reaching a limit near a tortuosity of 1.02.
proportion of birefringent collagen in the bright and dark views both changed significantly with sarcomere length (p < 0.0001 and p < 0.005, respectively) while the amount of overlap did not change (p > 0.25) (Fig. 6b).

Effect of depth

Since changes in collagen fiber configuration appeared to be tightly linked to changes in myocyte length, we tested whether the changes in collagen structure observed with depth were similar to previously measured transmural variations in sarcomere length (10, 28), namely that epicardial sarcomeres were longer than endocardial sarcomere. In this study, the epicardial regions had significantly longer sarcomeres than the endocardial regions (p < 0.02) and nearly longer sarcomeres than the midwall (p = 0.051) over all pressures (Table 3) (1.81 ± 0.02 μm, 1.80 ± 0.01 μm and 1.77 ± 0.02 μm for the unloaded pressure group at the epicardial, midwall and endocardial positions, respectively). In accordance with the inverse relationship between sarcomere length and amount of coil measured over all pressures, the collagen fibers were straighter near the epicardium than near the midwall or near the endocardiun (Table 3). This relationship was statistically significant for both tortuosity (p < 0.02) and the proportion of birefringent collagen observed in the bright-muscle view (p < 0.0001). In contrast, there was not a significant effect of depth on the proportion of collagen aligned oblique to the muscle (% Dark). There was no statistically significant interaction between pressure and depth for any of the parameters measured.

Changes in collagen independent of coiled structure

In addition to the proportion of birefringent collagen measured at each orientation, we measured the area fraction

<table>
<thead>
<tr>
<th>Depth</th>
<th>SL (μm)</th>
<th>Tortuosity</th>
<th>Bright</th>
<th>Dark</th>
<th>Overlap</th>
<th>Bright</th>
<th>Area fraction (%)</th>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Epi</td>
<td>1.863±0.023</td>
<td>1.050±0.017</td>
<td>83.7±3.6</td>
<td>36.7±5.4</td>
<td>15.8±2.1</td>
<td>4.75±0.72</td>
<td>2.67±0.29</td>
</tr>
<tr>
<td>Mid</td>
<td>1.836±0.020</td>
<td>1.068±0.015</td>
<td>77.0±2.5</td>
<td>41.0±4.3</td>
<td>14.2±2.2</td>
<td>3.49±0.54</td>
<td>2.37±0.42</td>
</tr>
<tr>
<td>Endo</td>
<td>1.828±0.030</td>
<td>1.070±0.016</td>
<td>76.3±2.7</td>
<td>40.2±4.3</td>
<td>13.1±1.8</td>
<td>2.97±0.36</td>
<td>1.93±0.27</td>
</tr>
</tbody>
</table>

| p-value | <0.05 | <0.02 | <0.0001 | NS | p = 0.06 | <0.0001 | <0.002 | <0.0001 |

Table 3 Summary of effect of transmural position on measured parameters (mean±SE). The means and variations include data for all pressure groups. Consistent with previous reports, epicardial sarcomeres were longer than endocardial sarcomeres. The coiled structure varied in parallel with epicardial collagen fibers straighter than endocardial ones, as indicated both by tortuosity and the collagen birefringence. Independent of the collagen structure, epicardial regions had more collagen than endocardial regions. Statistics performed with a two-way analysis of variance.
having less collagen than either the midwall or epicardial regions (Table 3). However, collagen area fraction in the dark-muscle view did not vary with pressure (2.6 ± 0.1 % at zero pressure vs. 2.5 ± 0.2 % at high pressure) or sarcomere length by regression (p = 0.98 and p = 0.70, respectively). However, two-way ANOVA showed that the medium pressure group had significantly less collagen than any other group (p < 0.003 for overall effect).

If these relationships for collagen area fraction as a function of depth and pressure were simply consequences of the change in collagen fiber configuration, one would not expect changes in total birefringent collagen area fraction relative to muscle. However, the transmural variation in total collagen area fraction paralleled trends both in bright-muscle and dark-muscle birefringent collagen area fractions with the epicardial regions having more birefringent collagen than either the midwall or endocardial regions (Table 3). Furthermore, significant increases in total collagen area fraction as a function of pressure or sarcomere length were observed both with regression (p < 0.02 and p < 0.001, respectively) and ANOVA (p < 0.0001). Area fraction of birefringent collagen in the high pressure group (5.6 ± 0.2 %) was greater than any other group (4.5 ± 0.2 % for zero pressure).

Fig. 6 (A) Mean collagen fiber tortuosity and (B) proportion of collagen as a function of myocyte sarcomere length. The decrease in tortuosity, increase in % bright and decrease in % dark as a function of sarcomere length suggest a tight coupling between the myocytes and collagen fibers.

Confocal microscopy

Examination of the unloaded collagen fiber with laser scanning confocal microscopy revealed a distinct 3D structure. When the 28 optical sections were examined as a rotating projection, the structure resembled a “twisted ribbon” with regions of planar waviness rotating around the fiber axis. The extended focus views of the fiber from the top of the section (identical to that observed with light microscopy) and the profile view (from vertical sections parallel to the fiber axis) demonstrate this behavior by revealing an inverse relationship between the most and least tortuous regions (Fig. 7). The most tortuous region in the top view is the straightest in the profile view and vice versa. This single example suggests that the 3D length of the fiber is substantially greater than the 2D length measured using light microscopy.

Discussion

The objective of this study was to quantitatively determine whether the collagen fibers stretched or merely straightened in the normal range of diastolic pressures. As the ventricle distended with passive load, sarcomeres gradually increased in length and the perimysial collagen
fibers became proportionately straighter. Therefore the collagen fibers do not suddenly uncoil at high left ventricular pressures as suggested previously (6), but rather demonstrate a continuous reduction in collagen fiber tortuosity over the normal diastolic range. Furthermore, the parallel changes in collagen fiber configuration and sarcomere length suggest a tight coupling between the myocytes and the surrounding collagen matrix.

Other studies have shown sarcomere lengths ranging from 1.85 - 2.4 \( \mu \text{m} \) over the diastolic range (8) contracting to 1.7 \( \mu \text{m} \) during systole (27). Our sarcomere lengths ranged from 1.69 - 1.96 \( \mu \text{m} \). The shorter sarcomeres were most likely caused by the embedding of the tissue in paraffin, which can induce 30 - 40% shrinkage (14). It is not possible to use the birefringence enhancing collagen stain, picrosirius red, in plastic embedded sections which have less shrinkage (18). However, the changes in sarcomere length as a function of LV pressure in these hearts were similar to those measured by Grimm et al. (8) (increased 0.10 \( \mu \text{m} \) and 0.19 \( \mu \text{m} \), respectively, from 0 to 24 mmHg).

They measured sarcomere lengths in cryosectioned rat hearts and found that sarcomeres gradually lengthened over low pressures, approaching a plateau at medium pressures, and finally reaching a maximum at 48 mmHg. In this study we did not prepare any hearts at >40 mmHg, but the trend towards a plateau began at approximately 15 - 20 mmHg. The sarcomere lengths in our study were consistently 11 ± 2% shorter than sarcomeres measured in cryosectioned rat hearts fixed at similar pressures by Grimm et al. (8). To confirm that the differences were indeed related to shrinkage caused by paraffin embedding, we measured sarcomere lengths in cryosection samples from separate rings for 3 of the experimental hearts. The sarcomere lengths were an average of 7% longer than the paraffin-embedded samples in the same hearts, which accounts for 65% of the difference between this data and that by Grimm and coworkers (8). Other potential sources of shrinkage include contracture either during the experiment or during fixation. The former possibility was reduced through the use of BDM (19). In the more than 1400 measurements of sarcomere length, contraction bands were not observed, suggesting that there was not localized contracture during fixation. Although we recognize that the sarcomere lengths are underestimated, the variations in sarcomere length and tortuosity were intended to be compared within and between the groups which were all prepared identically and presumably have similar degrees of shrinkage.

It was not possible to measure the collagen fiber tortuosity in the cryosectioned samples because of damage to the extracellular matrix during sectioning. If the shrinkage had altered the collagen fiber configuration similar to the change in sarcomere length, the unloaded collagen fibers may be straighter. However, since the 3D length is greater than the 2D length, the difference in tortuosity should have been smaller (~4% vs. the measured 7%), and the conclusion that the collagen fibers are gradually straightening and are not completely straight over the normal range of diastolic pressures (10 - 15 mmHg) would still be correct. Additionally, Robinson et al. (26) observed collagen fibers with similar degrees of coil in papillary muscle. Also, numerous studies have identified coiled perimysial collagen fibers in the unloaded configuration using SEM and silver staining (2, 6). It is possible, however, that at the highest pressures (40 mmHg), these fibers were straight. The only way the major conclusions of this study could have been influenced by the preparation would have been if shrinkage differentially altered the low or high groups in only one of the variables measured. This is unlikely because sarcomeres from the unloaded and high pressure hearts had similar levels of paraffin-induced shrinkage and changes in collagen structure were confirmed with two independent methods.

Robinson et al. (26) examined the collagen structure of the coiled perimysial fibers in unloaded and stretched (15%) papillary muscles using scanning electron micros-
scopy and also found a decrease in the degree of coil with load. Factor et al. (6) examined the collagen structure in the silver-stained LV tissue at high ventricular pressures (30, 70 and 100 mmHg) and found that all the fibers were completely straight and/or ruptured at 100 mmHg. However, they did not quantify these structural changes. Surprisingly, they found that the tissue fixed at 30 mmHg (in the high range of the current study), revealed variable degrees of coil with some fibers highly coiled and some fibers virtually straight. These discrepancy between both the current study and the papillary muscle study (26) compared with the study by Factor et al. (6) may reflect differences in the methods of fixation (i.e., they utilized immersion fixation of the intact ventricle). The results of the current study provide the first quantitative examination of the changes in the structure of the large perimysial collagen fibers over the normal working range of LV pressures at both low and high ventricular stiffnesses.

Collagen fiber configuration was measured with two complementary but independent methods which both lead to the conclusion that the collagen fibers were gradually uncoiling. Tortuosity decreased as left ventricular pressure and sarcomere lengths increased indicating that the collagen fibers straightened. In papillary muscle, Robinson et al. (26) used a 3D representation of tortuosity that they called the convolution index. It was estimated as 1.4 for a single coiled fiber in the unloaded tissue based on the diameter and the pitch of the coil. The equivalent 2D tortuosity would be 1.20, which was within the range of values we observed. A 15% stretch of the papillary muscle decreased the degree of coil, but it did not become completely straight. Their convolution index estimate for the loaded tissue was 1.2 which would yield a 2D tortuosity of 1.10. Factor et al. (6) found that the LV needed to be loaded to 70 mmHg to find completely straight collagen fibers.

The birefringence of the collagen at two unique orientations was quantified to assess the anisotropic organization. As ventricular load and sarcomere length increased, more regions of the fibers became aligned with the long axis of the collagen fibers (and hence the muscle fiber axis) yielding an increase in collagen oriented parallel to the muscle fiber and a decrease in collagen oriented obliquely. Canham et al. (1) used a similar technique for examining collagen fiber structure in cerebral blood vessels and found that an unloaded vessel had collagen fiber orientations consistent with sine waves that tended to straighten with load.

In the unloaded heart, the perimysial collagen fibers that run parallel to the muscle fibers appear wavy when they are viewed parallel to the myocytes axis (i.e., parallel to the epicardial tangent plane or circumferential midwall fibers from an equatorial cross-section) indicating that these fibers may be a coil. Laser scanning confocal microscopy allows optical sectioning and 3D reconstruction of these fibers in picrosirus red stained sections (5). 3D reconstruction of the geometry of the single picrosirus-stained collagen fiber revealed that it did not have a coiled appearance as described by Robinson et al. (26), but rather that of a twisted ribbon. In any case, the 3D path of the collagen fiber was greater than the 2D length estimated in the current study suggesting that the potential of the tissue to elongate before stretching the collagen fibers is greater than the 9% indicated by the unloaded tortuosity. The advantage of a tortuous structure in terms of mechanics is that it can exhibit both low and high stiffnesses in a single structure thereby allowing the myocytes and sarcomeres to increase in length over a large range without danger of overstretching them. Therefore, this "design" of the myocardium appears to be similar to that of other collagen containing tissues which undergo large deformations (skin, intestine, blood vessels) (1, 12, 21, 24).

It is clear that the perimysial fibers are straightening during stretch of the myocytes, however it is not obvious whether the fibers are being stretched as they uncoil. We can approximate collagen fiber length by multiplying tortuosity by an estimate of midline lengthening (such as sarcomere length). If the collagen fibers were stretching as the ventricle dilated or if the sarcomeres reached their maximal limit before the collagen fibers stopped straightening, this measure would increase. In this study, the collagen fiber length does not appear to be changing with LV pressure (shown in Fig. 8), suggesting that the col-

![Fig. 8 Changes in collagen fiber length (represented by the multiplication of sarcomere length by tortuosity for each section) as a function of LV pressure. As LV pressure increases, the collagen fibers do not appear to be increasing in length. Therefore, most of the change in collagen fiber configuration appears to be due to uncoiling rather than direct stretch of the collagen fibers.](image-url)
lagen fibers are not being stretched. Rather, the majority of the change in collagen fiber configuration is due to uncoiling. Since the maximal sarcomere length corresponds to near straightening of the collagen fibers, these results suggest that the sarcomere extension is primarily limited by the perimysial collagen fibers in the normal range of diastolic pressures (9). These conclusions can be further supported by models of the collagen fiber architecture that can accurately model the resting myocardial mechanics by describing the collagen fibers as inextensible rods subject to bending and torsion (17).

In addition to measuring changes in collagen and myocyte structure that occurred with load, we measured variations with transmural position. Sarcomere length variations with depth were similar to those observed previously, with epicardial sarcomeres longer than endocardial sarcomeres (8, 28). We found comparable relationships for the collagen fibers with straighter fibers on the epicardium than endocardium. These differences may be the result of residual stress as identified recently by Rodriguez et al. (27) for sarcomere length. Functionally, the straighter collagen fibers may also give rise to stiffer tissue in the epicardial regions than the endocardial regions.

Changes in collagen density independent of the coiled structure were also observed. Because the collagen fibers straighten with load and transmural position, one should expect to see differences in the birefringent collagen area fraction measurements consistent with this uncoiling (i.e., increases in bright-muscle collagen area fraction and decreases in dark-muscle collagen area fraction as the fibers become straighter). While the expected transmural variation was observed in the bright-muscle collagen area fraction measurements, dark-muscle collagen area fraction actually increased as the collagen fibers straightened (1.78 % birefringent collagen area fraction/100 % wall thickness, respectively, from endocardium to epicardium). Since the images could be joined to examine the total area fraction of birefringent collagen, it was also possible to elucidate differences independent of the coiled structure. In this case, epicardial regions had more collagen than the endocardial regions for total collagen area fraction measurements (1.69 % birefringent collagen area fraction/100 % wall thickness, from endocardium to epicardium). This variation was similar to biochemical measurements made at similar locations from rat hearts (3). These transmural gradients in collagen density may indicate that the epicardial regions are more tightly coupled and stiffer than the endocardial regions.

The cardiac collagen matrix is characterized by a complex hierarchy (2, 29); however, only the configuration of the large perimysial fibers was examined here. Several mathematical models of diastolic myocardial mechanics have suggested that the myocytes contribute little to the resting stress-strain behavior of the tissue (11, 20). In particular, we developed a model of the myocardial collagen structure (17) based on the current observations of gradual straightening of inextensible coiled fibers without a contribution from the myocytes. The model suggests that these perimysial collagen fibers may be a primary determinant of the nonlinear material properties of the myocardium in the direction of the myofibers (17). In contrast, recent results by Linke et al. (15) suggest that the intermediate filament titin may be the major component of the myocardial stiffness in the low range of LV pressures. There are also other components of the myocardium, including the rest of the collagen matrix that should also play important roles in determining myocardial stiffness. In particular, they should be important for the behavior in directions other than the fiber direction (i.e., crossfiber or shear). The evaluation of the relative contribution of the perimysial collagen fibers, titin, other components of the collagen matrix and other myocardial proteins still remains unresolved and requires future study. The results presented in the present article suggest that the perimysial collagen fibers do not contribute by stretching during diastole, but rather gradual straightening, and may contribute to stiffness in a nonlinear fashion.

Finally, although it is well established that the cardiac extracellular matrix can undergo remodeling with disease, most evaluations have been characterized by a single measurement or a non-quantitative examination of the collagen structure (3, 13). This study implies that collagen fibrillar organization (i.e., tortuosity and local collagen fiber orientation) contains additional factors that should be evaluated in these pathophysiologic states to fully understand the functional implications.
References