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Modulation of collagen fiber orientation by strain-controlled enzymatic degradation

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ABSTRACT

Collagen fiber anisotropy has a significant influence on the function and mechanical properties of cardiovascular tissues. We investigated if strain-dependent collagen degradation can explain collagen orientation in response to uniaxial and biaxial mechanical loads. First, decellularized pericardial samples were stretched to a fixed uniaxial strain and after adding a collagen degrading enzyme (collagenase), force relaxation was measured to calculate the degradation rate. This data was used to identify the strain-dependent degradation rate. A minimum was observed in the degradation rate curve. It was then demonstrated, for the first time, that biaxial strain in combination with collagenase alters the collagen fiber alignment from an initially isotropic distribution to an anisotropic distribution with a mean alignment corresponding with the strain at the minimum degradation rate, which may be in between the principal strain directions. When both strains were smaller than the minimum degradation point, fibers tended to align in the direction of the larger strain and when both strains were larger than the minimum degradation, fibers mainly aligned in the direction of the smaller strain. However, when one strain was larger and one was smaller than the minimum degradation point, the observed fiber alignment was in between the principal strain directions. In the absence of collagenase, uniaxial and biaxial strains only had a slight effect on the collagen (re)orientation of the decellularized samples.

Statement of Significance

Collagen fiber orientation is a significant determinant of the mechanical properties of native tissues. To mimic the native-like collagen alignment in vitro, we need to understand the underlying mechanisms that direct this alignment. In the current study, we aimed to control collagen fiber orientation by applying biaxial strains in the presence of collagenase. We hypothesized that strain-dependent collagen degradation can describe specific collagen orientation when biaxial mechanical strains are applied. Based on this hypothesis, collagen fibers align in the direction where the degradation is minimal. Pericardial tissues, as isotropic collagen matrices, were decellularized and subjected to a fixed uniaxial strain. Then, collagenase was added to initiate the collagen degradation and the relaxation of force was measured to indicate the degradation rate. The V-shaped relationship between degradation rate and strain was obtained to identify the minimum degradation rate point. It was then demonstrated, for the first time, that biaxial strain in combination with collagenase alters the collagen fiber alignment from almost isotropic to a direction corresponding with the strain at the minimum degradation rate.

1. Introduction

Collagen is a major structural element of extracellular matrix (ECM) and confers mechanical and structural integrity to most biological tissues. A well-organized collagen matrix is therefore one of the requirements of a tissue engineered substitutes to withstand in vivo forces [1–6]. In order to create such an engineered tissue, a thorough understanding of the underlying mechanisms driving collagen fiber orientation is essential.

It has been assumed that the orientation of collagen fibers in the ECM is related to the cell orientation: either new collagen fibers are synthesized along the main direction of the cells [7,8], or the cell traction forces realign the fibers [9–11]. However, another theory
suggests that collagen fiber anisotropy is guided at the molecular level by strain-dependent enzymatic degradation [12,13]. Packing of the collagen molecules while exposed to mechanical loading decreases their susceptibility to cleavage by enzymes. Based on this theory, mechanical strain can modulate the enzymatic degradation of collagen fibers and create preferential fiber alignment by selectively retaining strained fibers, resulting in strain-stabilization [12–14].

Collagen synthesis and degradation is an important feature of a normal tissue undergoing growth and morphogenesis. A number of enzymes, notably the matrix metalloproteinases (MMPs), are involved in the degradation of collagen fibers [15]. In this process, the collagen triple helix α-chains are unfolded and the denatured chains are exposed to further degradation [15,16]. Bacterial collagenase, referred to as MMP, has been used as a collagen-degrading enzyme in many in vitro studies [13,14,17].

To investigate the enzymatic degradation of collagen under strain, Huang and Yannas [17] developed an in vitro mechanochemical experiment to quantify the degradation rate of collagen fibers in reconstituted collagen gels, induced by bacterial collagenase, as a function of the applied uniaxial strain. They reported that with increasing the strain, collagen fiber degradation rate first decreased and then increased, resulting in a U-shaped curve and a strain level where the degradation rate was minimum.

Based on their results, we hypothesize that the minimum in degradation rate may explain the alignment of collagen fibers in biaxially loaded samples. So far, studies conducted to evaluate the effect of strain on collagen fibers have concluded that by applying a uniaxial strain to a collagen fiber matrix, the fibers that are not in the direction of the strain are degraded, while strained fibers are retained [12–14]. However, the effect of biaxial straining on collagen alignment has not been studied. We hypothesize that collagen fibers, when they are biaxially strained, preferentially align in the direction with the lowest degradation rate.

In the present study, by application of a range of uniaxial tensile strains to a biologically relevant tissue with a relatively isotropic collagen matrix (a decellularized pericardium), we first identified the strain at which the degradation rate was minimum. Next, to test our hypothesis, samples were subjected to biaxial tensile loads and a uniform concentration of collagenase, while strained samples without collagenase served as controls. Collagen fibers were visualized using second harmonic generation (SHG), which is a high resolution microscopy technique to visualize non-centrosymmetrical biological molecule structures such as collagen [18]. Collagen orientation was quantified for all samples and the fiber strains as a function of fiber angles were calculated to evaluate to what extend the fibers tend to align in the direction of the minimum degradation rate.

2. Materials and methods

2.1. Specimen preparation

Native pericardial samples of one-year old pigs (n = 12) isolated from fresh hearts were collected from a slaughterhouse. After the sacs were excised from the surrounding tissues and washed three times in PBS (Sigma-Aldrich), they were decellularized as described before [19]. Briefly, samples were incubated in PBS supplemented with 0.25% Triton X-100 (Merck, Darmstadt Germany), 0.25% sodium deoxycholate (SD, Sigma-Aldrich), and 0.02% EDTA (Sigma-Aldrich) at 37 °C. Then, samples were washed twice in PBS, followed by removing the nucleic remnants by incubating in a nuclease digestion solution of 50-mM TRIS-HCl buffer (Tris (hydroxymethyl)-aminomethane (Tris) pH 8.0, supplemented with 100 U/ml Benzonase® (25 units/ml, Novagen, Madison WI USA) and 1 mmol/l of MgCl2 (Merck) at 37 °C for 5–8 h. Subsequently, they were treated overnight with a nuclease digestion solution containing 80 U/ml Benzonase®. The solution was changed by a 20 U/ml Benzonase® for 5–8 h. Finally, to remove cellular remnants, samples were incubated for 24 h at 4 °C. They were sterilized in 70% EtOH (VWR international S.A.S. Fontenay-Sous-Bois, France) for 30 min afterwards and stored in PBS at 4 °C.

2.2. Experimental design

To quantify the degradation rate of a pericardial tissue as a function of strain, 5 mm × 5 mm samples (n = 26) were uniaxially stretched at a strain rate of 1 mm/min using a BioTester (Biaxial test system; CellScale, Waterloo, Canada) to a fixed extension in a bath of PBS at a constant temperature of 37 °C (Fig. 1A, B). After a relaxation period of 60 min, the PBS was replaced with 45 μg/ml collagenase (Sigma Life Science, St. Louis, Missouri) in PBS. By adding the enzyme and initiating the degradation, the force decayed continuously for six additional hours. However, the force remained constant when samples (n = 3) were not exposed to the collagenase. The degradation rate was calculated based on fitting the measured force (f) to the degradation part of the force-time graph [17]:

\[
f = f_0 e^{-bt}
\]

(1)

The constant b represents the enzymatic degradation rate of collagen fibers and f0 is the initial force when the degradation starts. This process is shown schematically in Fig. 1C. To determine the relationship between strain magnitude and degradation rate, individual samples were subjected to strains of different magnitudes (up to 50%) and the degradation rate of each sample was calculated. Finally, a bi-linear fit was applied to all data and the strain level corresponding to the lowest degradation rate (εmin) was determined.

Next, different combinations of biaxial strains were applied. Following three general straining regimens, samples were loaded with strains (X, Y directions) that were 1) both smaller than εmin; 2) both larger than εmin; or 3) one smaller than εmin and the other larger than εmin. Equivalent to the uniaxial experiments, samples were relaxed for 60 min after they were stretched biaxially and were subsequently exposed to enzymatic degradation for 6 additional hours. At the end of the experiment, samples were washed in PBS and fixed with 4% paraformaldehyde for 1 h while attached to the tensile tester. Next, samples were released and kept in PBS for collagen visualization. Before releasing the samples, edges of the samples were marked to identify the orientation of the samples with respect to the strain directions.

2.3. Collagen orientation visualization and quantification

Collagen visualization was performed on a Zeiss LSM 510 Meta laser scanning microscope (Carl Zeiss, Germany) attached to an inverted Axiowert 200 motorized microscope (Carl Zeiss, Germany). To conduct SHG imaging, a mode-locked chameleon ultra 140 fs pulsed Ti-Sapphire laser (Coherent, USA) was used. Collagen SHG signal was collected after excitation of samples with laser pulses at 800 nm. To quantify the fiber orientation in microscopy images, an algorithm developed in Mathematica (Wolfram, USA) was used [20–22]. In short, coherence enhancing diffusion (CED) was applied to the images to remove the noises and create a flow-like fibrous pattern. Then, the local fiber orientations were calculated from the principal curvature directions in each pixel obtained from eigenvalues and eigenvectors of Hessian’s matrix [20]. Finally, a histogram was exported containing all fiber orientations data in the image. Furthermore, the mean angle (α) with respect to the vertical (Y) axis, and variance (σ²) of the fibers distribution were
obtained based on the following Eq. (2), for each image by fitting single or bimodal Gaussian curves to the histograms.

\[ f(x) = A_1 \exp[-(x - a)^2 / 2\sigma^2] \]

2.4. Fiber stretch quantification

In order to calculate the fiber stretch as a function of fiber angle (\( \Phi \)) in biaxially strained samples, the following equation was used [23]:

\[ \lambda = \sqrt{\varepsilon_0 \cdot F^T \cdot F \cdot \varepsilon_0} \]

where \( \lambda \) is the stretch ratio in fiber direction, \( F \) is the deformation tensor from the reference state to the final state and \( \varepsilon_0(\Phi) \) is the unit vector in the reference configuration in the direction of a collagen fiber with fiber angle \( \Phi \).

In order to compare the fiber strain values in the direction of the fiber mean angle in biaxially strained samples where the strain in one direction (\( \varepsilon_1 \)) was smaller and strain in the other direction (\( \varepsilon_2 \)) was larger than \( \varepsilon_{\text{min}} \), their degradation rates were calculated and deducted from the minimum degradation rate value, giving \( \Delta_1 \) and \( \Delta_2 \), respectively. Then, degradation rate ratios were calculated by dividing these two values (\( D = \Delta_2 / \Delta_1 \)).

2.5. Statistical analysis

Linear regression was applied to the strain values calculated from Eq. (3) versus degradation rate ratios (D) for biaxially strained samples (\( \varepsilon_1 < \varepsilon_{\text{min}} \) and \( \varepsilon_2 > \varepsilon_{\text{min}} \)) to evaluate the proximity of intercept of the regression line to the strain corresponding to \( \varepsilon_{\text{min}} \). Furthermore, the statistical differences between the measured mean fiber angles and the predicted mean fiber angles were determined using paired t-test. A P-value of 0.05 was considered statistically significant. Statistical analysis was performed using GraphPad Prism 5 software.

3. Results

3.1. Uniaxial experiments

The V-shaped degradation rate curve has a minimum at a strain of approximately \( \varepsilon_{\text{min}} = 20\% \) (Fig. 2). In general, collagen fibers were almost randomly oriented before straining (Fig. 3A and C). In the absence of collagenase, the main fiber direction was also in the loading direction, but the dispersity was higher, meaning that there were more fibers not coincident with the load axis (Fig. 3B). As an example, after applying 35% uniaxial strain and in the presence of collagenase, fibers were mainly distributed in the direction of the load axis (Fig. 3D).

3.2. Biaxial experiments

In order to compare the collagen fiber orientation before (Fig. 4A) and after (Fig. 4B) applying the biaxial strain, the central part of each sample was visualized and collagen orientation was quantified. As an example, Fig. 4 shows how collagen fiber orientation could be influenced by applying a biaxial load, with and without the presence of the collagenase, in this case of 15% and 28% (when collagenase was not applied, upper panel of Fig. 4B) and...
17% and 25% (when collagenase was added, lower panel of Fig. 4B) in X and Y direction, respectively.

Examples of the effect of the three different biaxial straining regimens on collagen fiber orientation in the decellularized pericardial tissues are shown in Fig. 5. For regimen 1 (strains both smaller than $e_{\text{min}}$), the preferred fiber orientation was toward the direction of the larger strain, which was closer to $e_{\text{min}}$ (Fig. 5A). For strains larger than $e_{\text{min}}$, the fiber orientation was toward the direction of the smaller strain, which was again closer to $e_{\text{min}}$ (Fig. 5B). However, when one strain was smaller and one was larger than $e_{\text{min}}$, the remaining observed collagen fiber orientation was in between the X and Y axis (the principal strain directions) in the direction where the fiber strain was closest to $e_{\text{min}}$ (Fig. 5C).

The strain value ($e_{m}$) of fibers in the direction of the mean angle ($\alpha$, Eq. (2)) can be calculated based on Eq. (3). The method used to calculate the degradation rate ratio (D) is illustrated in Fig. 6A. Fiber strains ($e_{m}$) for all samples ($n = 8$), in which one strain was smaller than $e_{\text{min}}$ and one larger than $e_{\text{min}}$, were calculated and plotted as a function of degradation rate ratio D (Fig. 6B). There were two mean fiber angles and thus two calculated strain values in two experiments. The linear regression line through all data points revealed that the strain along the mean fiber orientation was approximately 25%, which was close to the strain value corresponding to the minimum degradation rate value. Furthermore, the predicted fiber angles with respect to the Y axis obtained from Eq. (3) were compared with the measured fiber angles obtained from the microscopy images (Fig. 6C). Paired t-test indicated that there was no significant difference between measured and predicted fiber angles.

The mean angle of collagen fibers for biaxial strains smaller $e_{\text{min}}$ ($n = 3$) and biaxial strains larger than $e_{\text{min}}$ ($n = 2$) is summarized in Fig. 7. When both strains were smaller than $e_{\text{min}}$, collagen fibers...
were mainly aligned toward the direction of the larger strain. However, when both strains were larger than $e_{\text{min}}$, fibers tended to align in the direction of the smaller strain. No significant difference was found between predicted and measured fiber angles with respect to Y axis using paired $t$-test (Fig. 7B).

4. Discussion

Collagen fiber orientation is a significant determinant of the mechanical properties of the tissue. To mimic the native-like collagen alignment, we need to understand the underlying mechanisms that direct this alignment. In the current study, we aimed to control collagen fiber orientation by applying biaxial strains in the presence of collagenase. We hypothesize that the observed mean fiber orientation is in the direction of the fibers having the smallest strain-dependent degradation rate, because enzymatic degradation would preferentially remove fibers in other directions. To test our hypothesis, first we investigated whether there is a strain amount ($e_{\text{min}}$) at which the degradation rate is minimal by applying a range of uniaxial strains. It was challenging to collect many data points at high strains (above $\sim$40%) as the thin pericardial tissue could rupture at the gripper sites. Moreover, the force decay corresponding to the low strains (below $\sim$5%) could not be measured accurately due to limited sensitivity of the load cell and thus not included in the data points.

Based on the obtained $e_{\text{min}}$, decellularized tissues were exposed to 3 different groups of biaxial strains. When both strains were smaller than $e_{\text{min}}$ (first group) or larger than $e_{\text{min}}$ (second group), fiber orientation was pronounced toward the direction of the larger strain in the first group and the smaller strain in the second group as the degradation in these directions was closer to the minimum point. Whereas, when one strain was smaller and the other one...
was larger than $e_{\text{min}}$, collagen fibers had a preferred orientation toward a direction in between two axes and in close proximity to the direction where the fiber strain was equal to $e_{\text{min}}$. As pericardial tissues were very thin, performing successful experiments at which biaxial strains were both larger than $e_{\text{min}}$ (second group) were not very successful due to rupture of the tissue.

Huang and Yannas discovered that collagen degradation by collagenase was influenced by the uniaxial strain [17]. They found that the lowest degradation rate in a reconstituted collagen construct occurred when it was strained at 4%. Huang and Yannas assumed that by increasing the strain at low strain level, where the degradation rate decreases with strain, the porosity of the substrate decreases which results in less diffusion of enzyme into the fibers. However, an increase in the degradation rate with high strains might be due to the fact that the enzymatic attack sites in collagen molecules are only accessible after the fibers are stretched. Another explanation was that when a few fibers are broken down by enzymatic attack, stress is transferred to the neighboring fibers resulting in disruption of more fibers [17]. It has been shown that the thermal stability of collagen monomer improves when they are packed into fibrils due to the loss of configurational entropy of collagen molecules [24]. It is possible that the same mechanism applies when fibers are exposed to the mechanical loading. Bhole et al. (2009) suggested that applying...
strain to the collagen monomers can make the enzyme binding more difficult by changing the geometry of the binding sites. Moreover, it can make the cleavage of monomers more difficult by changing the locations of three alpha-chains [14]. Chang and Buehler also suggested that there are two vulnerable conformations of the collagen molecule cleavage sites: 1) micro unfolding conformation which happens when the strain can slow down the rate of cleavage, and 2) unwinding conformation which occurs when the strain can speed up the rate of cleavage [25].

In the present study the relationship between the strain and degradation rate during collagenase treatment was studied in decellularized pericardial samples. It was observed that the V-shaped curve describing this relationship was shifted to higher strain values compared to the curve reported by Huang and Yanonas, and the minimum degradation value was found around 20% strain. This shift of the curve might be due to the fact that the reconstituted collagen construct consists of relatively straight fibers. However, the pericardium consists of undulated fibers, meaning that a larger strain is needed to straighten the fibers and achieve strain-stabilization.

After obtaining the minimum degradation point, different combinations of biaxial strains in the presence of collagenase were applied to the pericardium samples in order to validate our hypothesis. In the unstrained configuration, the fiber distribution...
In conclusion, collagen fiber orientation was modulated by strain-controlled enzymatic degradation. When both strains were smaller or larger than $\varepsilon_{\text{min}}$, the preferred fiber orientation was in the direction of the strain with a lower degradation rate. However, when one strain was smaller and the other was larger than $\varepsilon_{\text{min}}$, fibers tended to align in the direction where the degradation rate was minimum, which is in between the principal strain directions. It was also shown that there was a certain amount of reorientation when the samples were biaxially strained in the absence of collagenase. Adding collagenase to the system may accentuate the reorientation process as cleavage of some fibers may ease reorientation of the others. More studies need to be conducted to reveal the underlying mechanism of the strain-controlled enzymatic degradation to indicate the contribution of reorientation in the final collagen fiber alignment. These results would help us to design well-structured and functional tissue engineered constructs. For instance, strain-stabilization requires the presence of degrading enzymes that can be delivered or activated via a scaffold. As such, it is an interesting route for novel in situ tissue engineering strategies that aim at using cell-free scaffolds.

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