Acta Biomaterialia 35 (2016) 118–126

Contents lists available at ScienceDirect

Acta Biomaterialia

journal homepage: www.elsevier.com/locate/actabiomat

Full length article

Modulation of collagen fiber orientation by strain-controlled enzymatic degradation

S. Ghazanfari [a], A. Driessen-Mol [a], C.V.C. Bouten [a,b], F.P.T. Baaijens [a,b,*]

[a] Department of Biomedical Engineering, Eindhoven University of Technology, Eindhoven, The Netherlands
[b] Institute for Complex Molecular Systems, Eindhoven University of Technology, Eindhoven, The Netherlands

ARTICLE INFO

Article history:
Received 13 July 2015
Received in revised form 17 December 2015
Accepted 22 February 2016
Available online 23 February 2016

Keywords:
Collagen fiber orientation
Collagen fiber degradation
Collagenase
Second harmonic generation
Tensile testing

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

* Corresponding author at: PO Box 513, 5600MB Eindhoven, The Netherlands.
E-mail address: f.p.t.baaijens@tue.nl (F.P.T. Baaijens).

http://dx.doi.org/10.1016/j.actbio.2016.02.033
1742-7061/

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, preferably 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-centrosymmetric 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 (Merck) 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]:

$$f = f_0 e^{-bt} \tag{1}$$

The constant b represents the enzymatic degradation rate of collagen fibers and f_0 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 (ε_{min}) 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 ε_{min}; 2) both larger than ε_{min}; or 3) one smaller than ε_{min} and the other larger than ε_{min}. 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 Axiovert 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 (σ^2) of the fibers distribution were