AFM Investigations of Collagen Fibril Structure in the Murine Adventitia

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Abstract

Collagen fibrils in the extracellular matrix (ECM) maintain mechanical properties of blood vessels and act as binding sites for various proteins. In the blood vessels, the adventitial layer is mainly comprised of fibrils of collagen types 1 and 3. Previous studies have shown that the amount of collagen deposited can be affected by the expression of certain collagen-binding proteins (CBP), mutations in the collagen polypeptide chains and additional factors accompanying pathogenesis of vascular diseases. Few studies have been done to elucidate the changes in structural features of these collagen fibrils. This study investigates changes collagen fibrils in the murine adventitia. Studies were performed ex-vivo on tissues extracted from mice lacking a CBP, discoidin domain receptor 1 (DDR1) and in a model of abdominal aortic aneurysm (AAA). Atomic force microscopy (AFM) was used to characterize the D-periodicity of collagen fibrils by measuring both the length of the D-period and the depth of the gap in the D-period. Our results show that the depth of the D-period in DDR1 KO mice was significantly greater than those in WT mice, but the length showed no significant difference. Collagen fibrils in AngII-infused ApoE models of AAA exhibited both normal and abnormal fibril characteristics. No significant differences in the depth or length of D-period was observed in normal collagen fibrils in AAA models as compared to saline-infused controls. However, the abnormal collagen fibrils in AAA revealed a complete lack of D-periodic depth as compared to normal fibrils. These results make the case that changes in the depth of D-periods in the collagen fibril a manifestation of the ECM environment and could be used to understand the pathogenesis of the disease.

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Publications

1. "Collagen Fibril Ultrastructure in Mice Lacking Discoidin Domain Receptor 1". Jeffrey R Tonniges, **Benjamin Albert**, Joan Y Lee, Edward P, Calomeni, Xiaokui Mo, Susan E. Cole and Gunjan Agarwal. *Microscopy and Microanalysis***,** 2016**,** 22 (03), 599-611.

Presentations

1. "Modification of collagen ultrastructure by DDR1 impacts matrix mineralization". Jeff Tonniges, **Benjamin Albert,** Yuping Li, Conrado Aparicio and Gunjan Agarwal. ASMB National Meeting, October 2014, Cleveland, OH.
2. "Ultrastructural Imaging of Collagen Fibrils in Mouse Model of Abdominal Aortic Aneurysm". Jeffrey R Tonniges, **Benjamin Albert**, Edward Calomeni, Chetan Hans and Gunjan Agarwal. Microscopy and Microanalysis, July 2016, Columbus, OH (**awarded best poster**).
3. "Collagen Ultrastructure in the Aortic Wall of DDR1 Mice". **Benjamin Albert**, Jeffrey Tonniges, and Gunjan Agarwal. Denman Undergraduate Research Forum, March 2014, Columbus, OH, March 2014.
4. "Effect of Collagen Fiber Structure on Cell-Matrix Interactions". **Benjamin Albert**, Jeffrey Tonniges, and Gunjan Agarwal. Fall Undergraduate Student Poster Forum, September 2014, Columbus, OH.

Awards/Fellowships

1. OSU Undergraduate Research Office Summer Research Fellow, 2014
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3. OSU Biomedical Engineering Undergraduate Research Achievement Award, 2016

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Introduction

Collagen type 1 is the most abundant component of the extracellular matrix (ECM) found in a number of solid tissues in mammalian species. It provides structural integrity and plays a role in dictating cell-matrix interactions. The ECM of blood vessels is comprised of proteins that allow for the elasticity and stiffness that the vessels require in order to be stable and adapt to pressures exerted by the blood circulation. Blood vessels rely on collagen to maintain the structure of the tunica adventitia layer (also known as tunica externa), which resides in the outer region of the vessel, furthest from the lumen (Figure 1). This adventitial layer, in both arteries and veins, is mostly comprised of collagen types 1 and 3 and provides the support that the vessel needs to maintain its shape and function (Bou-Gharios et al. 2004).

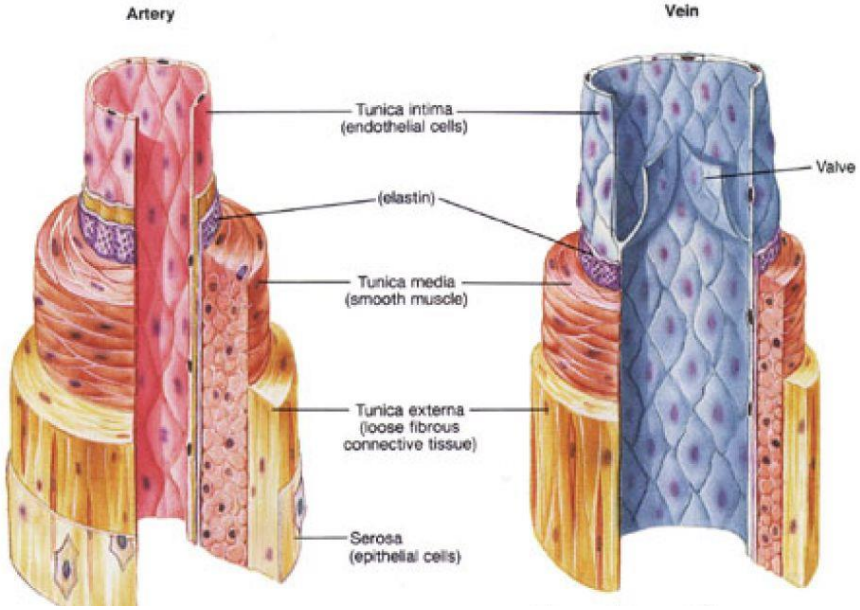

Figure 1: Representation of the composition and orientation of the layers in arteries and veins (Adapted from Fox, 2013)

Type 1 collagen is secreted from the cells as 'procollagen', which is a triple helical molecule with a rod-like morphology and globular propeptide domains flanking its ends. The collagen triple helix consists of three α-chains with Gly-X-Y repeats where X and Y are frequently prolines and hydroxyprolines. Upon secretion from the cells, the propeptide ends of the procollagen are cleaved by procollagen N-proteinases (Ricard-Blum, 2011). The triple helical structure that remains is known as 'tropocollagen'. Collagen fibrils are formed via tropocollagen molecules that are arranged in parallel with an offset between the ends of adjacent molecules (Orgel et al, 2011). These offsets or 'stagger' create the gaps and overlaps structure characteristic of a collagen fibril. Figure 2 represents a segment of a collagen fibril and the particular arrangement of tropocollagen that forms the fibril.

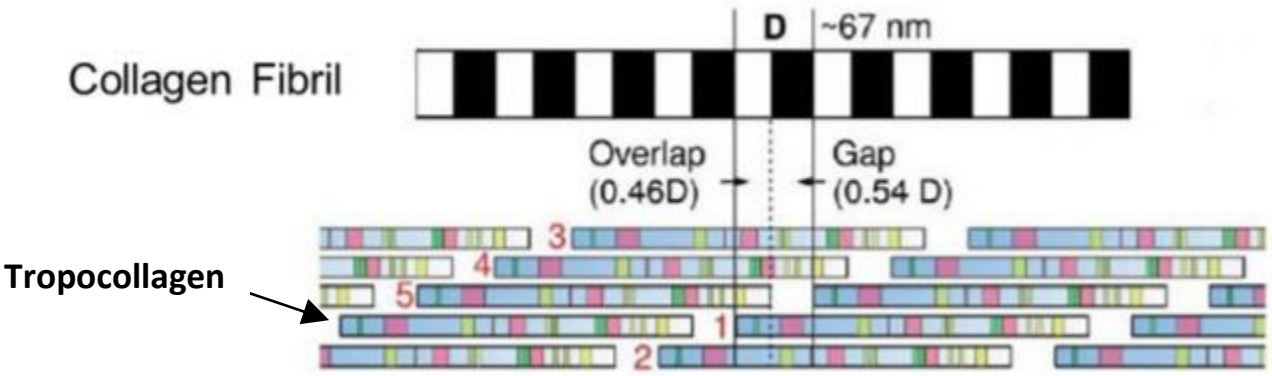

Figure 2: Characteristic formation of gap and overlap regions of the collagen fibril (Image adapted and modified from Orgel et al., 2011).

The collagen fibril structure can be perturbed by several factors such as mutations in the α-chain, incomplete cleavage of propeptides, or abnormal expression of certain collagen binding proteins (CBPs). Collagen mutations have been shown to affect the quantity and quality of collagen in the musculo-skeletal system in diseases such as osteogenesis imperfecta and Ehlers-Danlos syndrome (Ricard-Blum, 2011). However, much less is known on how the collagen fibrils

are affected in the vessel wall in health and disease. This is important as changes in the collagen fibril structure may alter the biomechanics of the vessel wall as well as expose or hide binding sites for other CBPs which may be a cause or an effect of vascular pathologies characterized by collagen remodeling like atherosclerosis, aneurysm, vascular dissections, and thrombosis (Ricard-Blum, 2011).

This study focuses on the effect of a CBP in modulating collagen fibril structure. Discoidin Domain Receptor 1 (DDR1) is a widely expressed CBP that is known to impact the quantity and quality of collagen fibrils in-vitro (Figure 3). The extracellular domain (ECD) of DDR1 has been shown to bind to collagen types 1-3 (Vogel et al. 1997) and the DDR1 binding site on the collagen is understood to be located in the gap region of collagen fibrils (Agarwal, 2016).

Earlier studies from our laboratory have shown that the DDR1 ECD binds to overlapping collagen monomers and disrupts collagen fibrillogenesis (Agarwal et al., 2007). Transmission

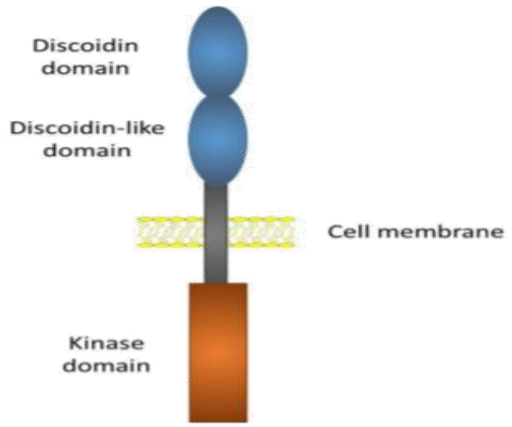

Figure 3: Schematic of discoidin domain receptor with respect to the cell membrane (Adapted from Tonniges et al., 2016a)

electron microscopy (TEM) of collagen fibrils formed in the presence of DDR1 showed a disruption in the D-periodic structure of collagen fibrils as compared to fibrils formed without DDR1 (Agarwal, 2016). However, whether there are any changes in the structure of the collagen

fibrils in-vivo has yet to be elucidated. This is especially important as DDR1 is known to be dysregulated in diseases such as atherosclerosis and fibrosis (Vogel et al, 2006).

Collagen fibril structure in-vivo is typically characterized by transmission electron microscopy (TEM). TEM is useful for measurements of features that lie along the lateral direction with respect to the path of the electrons. However, this method is unable to measure features which lie along the axis of the electron optics. Another method of quantifying the structure of collagen fibrils is through atomic force microscopy (AFM). AFM is useful for imaging topographical features of histological samples since the surface features can be measured in the x and y planes, as well as the z plane. Collagen fibrils and their banded structure can be imaged with AFM when the fibrils are parallel to the plane of the sample. AFM has been used in previous studies to characterize the length and depth of D-periods in collagen fibrils in the cornea and sclera (Table 1). From these previous in-vivo studies of collagen, we see that the length of the D-period remains relatively constant between the cornea and sclera, but the depth varies widely between the two tissue types. These results are indicative of little variation in the distance of the gap between tropocollagen ends and more variation in the organization of staggered tropocollagen in the gap region (Agarwal, 2016). Measurement of both the length and depth allows for greater insight into the structural changes in the collagen fibril that may occur in different studies.

Table 1: Summary of D-period depth measurements done using AFM on various eye tissues. Data shown are for control groups if applicable for the particular study.

	Species	Tissue	Length (nm)	Depth (nm)
Yamamoto et al., 1997	**Bovine**	Cornea	63.9±0.5	2.8±0.9
		Sclera	65.4±0.7	5.5±0.7
Miyagawa et al, 2000	**Murine**	Cornea	64.78±4.50	2.2±0.76
		Sclera	61.43±7.25	3.53±1.09
Miyagawa et al, 2001	**Human**	Cornea	68.65±6.44	2.38±0.82
		Sclera	66.25±5.32	5.48±0.1.23

To investigate the ultrastructure of collagen fibrils, this study used aortic sections from murine models. The collagen fibril length and depth of D-period in the adventitia of DDR1 knockout (KO) mice was compared to that of their wild-type (WT) littermates. Secondly, the collagen fibril length and depth of D-period in murine models displaying symptoms of abdominal aortic aneurysm (AAA) was compared to tissue from control mice. These studies provide insights into effective quantification of collagen fibril ultrastructure and in health and disease.

Materials and Methods

Mouse models

Discoidin Domain 1 Knockout (DDR1 KO) Mice were generated by Lexicon Pharmaceuticals (Woodlands, Texas, USA) via homologous recombination. These heterozygous mice were purchased through the Texas Institute of Genomic Medicine. DDR1 KO mice and their wild type (WT) littermates were obtained through breading of DDR1+/- heterozygous mice. Mice were genotyped using the methods described in Tonniges et al., 2016a. Tissue from age and gender-matched WT/KO littermates was used for the studies.

Abdominal aortic aneurysm (AAA) mouse models were generated by infusion of Angiotensin-II (AngII) in the laboratory of Dr. Chetan Hans at the Nationwide Children's Hospital (NCH). Male ApoE knockout mice (C57BL/6J background, Jackson Laboratory, Bar Harbor, ME) of age 8-10 weeks were infused subcutaneously with either AngII (1000 ng/min per kg) or saline using a mini-osmotic pump (model 2004; Alzet, Cupertino, CA). Mice received the infusion for 28 days. The first group of mice (Group 1) were sacrificed when the infusions of AngII/saline were stopped. A second group of mice (Group 2) were sacrificed 14 days after the end of the infusions.

Aorta Extraction

Thoracic aortas were excised from the mice within 30 minutes after sacrifice. The excised portion of the aorta occurred superior to the diaphragm and inferior to the left subclavian artery. Aortas were removed of any adipose tissue and embedded in optimal cutting

temperature (OCT) through flash freezing in liquid nitrogen. Poly-L-Lysine (EMD Millipore, Billerica, MA, USA) coated glass cover slips were prepared and five micron thick sections of the aorta were cryo-sectioned onto the coverslips. Aortas were then stored at -20°C until used. Aortic sections ready for use were washed three times with phosphate-buffered saline (PBS). Then samples were washed twice with ultrapure water and incubated for 1 hour with ultrapure water. The aorta sections were then air-dried overnight.

Atomic Force Microscopy

Glass coverslips with the prepared aortic sections were adhered to magnetic stubs for use in the Multimode AFM with a JV scanner and Nanoscope IIIa controller (Bruker Inc., Santa Barbara, CA, USA). A reflected light module attached above the AFM head enabled visualization of the aortic section and helped identify the adventitial and medial regions. AFM imaging was done in tapping mode in ambient air using NSC15 cantilevers (Micromasch, Estonia) that had a spring constant of 40 N/m. Images were captured in 512 lines per scan and a frequency of 2 Hz at a scan angle of zero degrees.

For both DDR1 KO/WT mice and AngII and saline infused ApoE KO mice the length of each period and depth of the D-periodicity of collagen fibrils in the adventitia was measured in fibrils that were within ±15° of the axis in which the AFM head scans. The length and depth of were measured using the geometric method described in Figure 4. Images were generated from mice pairs and analyzed using WSxM software (Horcas et al., 2007). Five periods were measured from at least five fibrils for each mouse included in the analysis.

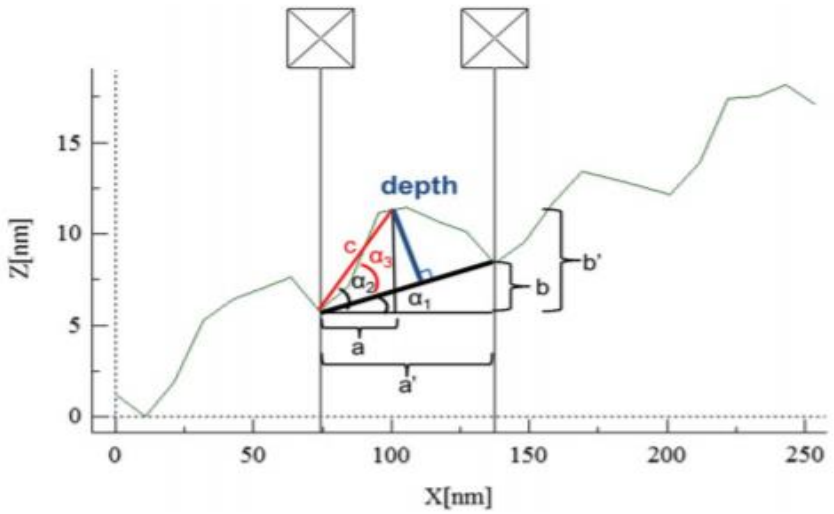

Figure 4: Schematic showing measurements used to calculate length and depth of D-period. Lengths a, a', b, b' and angles α1 and α2 were directly pulled from WSxM software. c and α3 were then found using trigonometric methods from the values already calculated. Length (bold black line) was found with cos(α1)/a'. Depth (bold blue line) was found with c*sin(α3). (Adapted from Tonniges, et al. 2016b)

In-vitro Reconstituted Collagen

Collagen type I fibrils in the form of PurCol collagen I (advanced BioMatrix, San Diego, CA, USA) were reconstituted in-vitro in neutral buffer conditions. Two different solutions were prepared: one with DDR1-Fc protein (R&D Systems, Minneapolis, MN, USA) and one without the protein. Solutions were then kept at 37^{o}C for 24h. Collagen fibrils formed in the solution were immobilized on freshly cleaved mica, washed with water, air-dried, and imaged with AFM.

Results

Effect of DDR1 on Collagen Fibril Structure

Quantifying the structure of the collagen fibrils through AFM utilized measurements of the length of the fibrils D-period (D-spacing) and the depth of the gap period within the D-period. The D-period banded structure of the collagen fibrils was clearly visible in both DDR1 KO and WT mice. Examples of images from the two groups are shown in Figure 5(A, B). Measurements of the length of D-period found that there is no significant difference between the DDR1 KO (67.5 ± 3.8 nm) and WT (67.0 ± 1.8 nm) (Figure 5(C)). These results were confirmed using TEM and SEM measurements (Tonniges et al., 2016b). Measurement of the depth of D-period displayed a significant difference (p=0.030) between DDR1 KO (3.6 ± 0.6 nm) and WT (3.0 ± 0.4 nm) (Figure 6). A summary of these measurements can be found in Table 2 and examples of the fibril backbone and height profile can be seen in Figure 6.

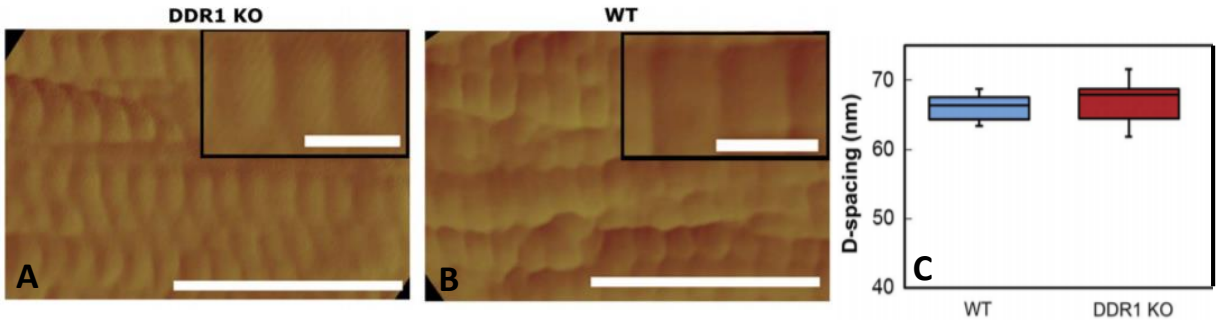

Figure 5: Characteristic AFM image of the banding structure of DDR1 KO (A) and WT (B) adventitial collagen fibrils. Outer scale bar is 225 nm and the inset scale bar is 100 nm for both (A) and (B). There is no significant difference in the length of the D-period (D-spacing) (C). (Adapted and modified from Tonniges, et al. 2016b)

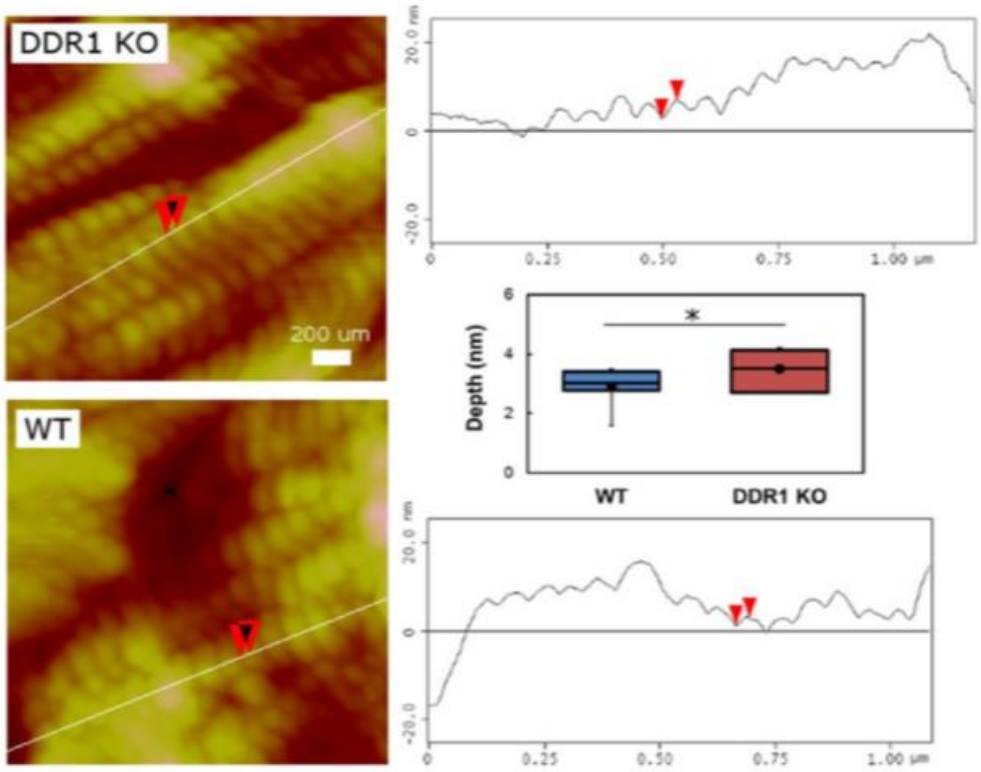

Figure 6: Characteristic collagen fibril backbone height profile for both DDR1 KO and WT mice and comparison of depth measurements (Adapted from Tonniges et al., 2016b)

In-vitro Reconstituted Collagen

Collagen fibrils that were reconstituted in the presence of DDR1-Fc showed no banding pattern along their length and also had no measureable depth of D-Period (Figure 7). Fibrils reconstituted in the absence of DDR1-Fc exhibited a D-length similar to fibrils of the in-vivo studies but a smaller D-depth.

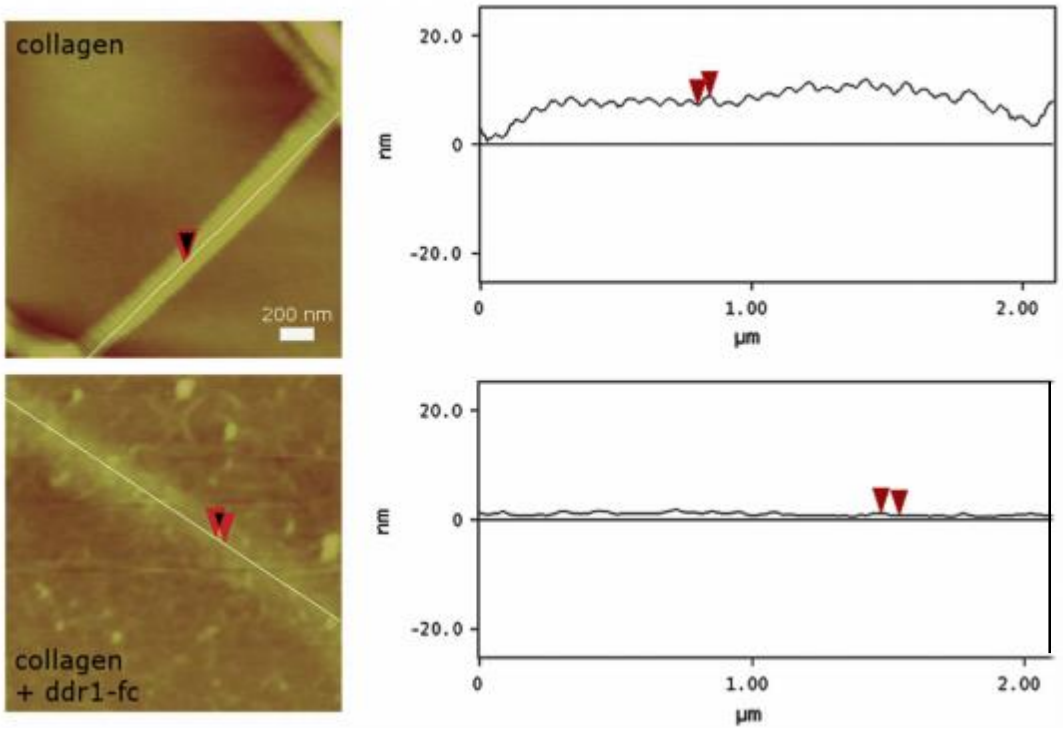

Figure 7: AFM images of reconstituted collagen fibrils in the absence of DDR1-Fc (Top) and in the presence of DDR1-Fc (Bottom) next to the AFM height profile of the fibril backbone (Adapted from Tonniges et al, 2016b).

Effect of AAA on Collagen Fibril Structure

Measurements of the collagen fibril structure on the AngII and saline-infused mice were done to evaluate the length and depth of the of the D-period. Both groups of mice displayed "normal" banding structure of collagen, characterized by smooth transitions between gap and overlap regions of the fibril. However, the AngII infused mice were also characterized by a subpopulation of "abnormal" fibrils, characterized by interrupted or disrupted periodicity. These differences in collagen appearance are shown in Figure 8 for the three groups of mice.

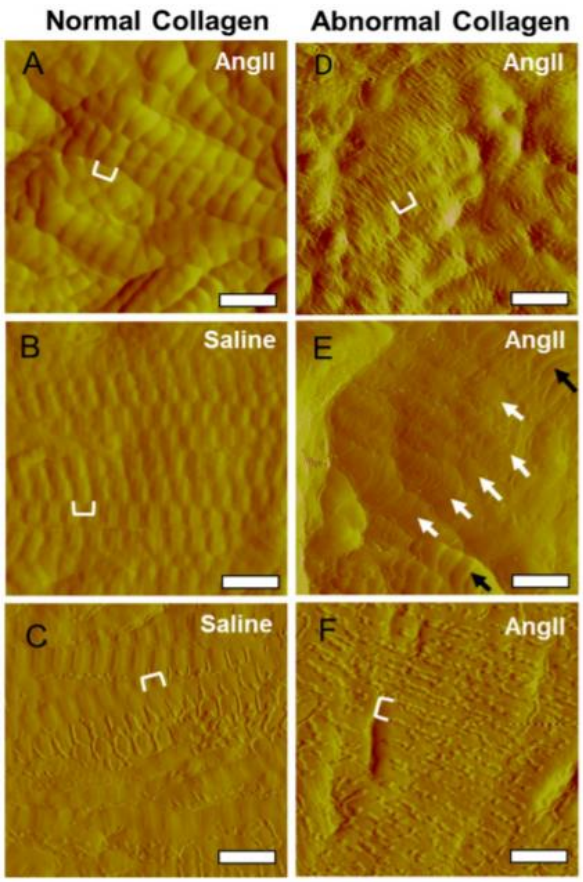

Figure 8: Collagen banding patterns found in ApoE KO mice infused with AngII (A, D, E, F) or saline (B, C). Characteristic banding structure was observed in both AngII (A) and saline-infused mice (B). Disrupted and incompletely formed banding structure was also seen in both types (C, D, E, F). White brackets represent periods of ~67 nm, black arrows show normal fibrils, and white arrows indicate abnormal fibrils. Each scale bar is 200 nm. (Adapted from Tonniges, et al. 2016b)

There were no significant differences between the lengths of the D-period for any of the three groups of mice: AngII Group 1 (62.8 ± 2.6), AngII Group 2 (67.0 ± 2.4 nm), and saline (67.2 ± 4.4 nm) for normal as well as abnormal fibrils (when measurable). Of the depths that were able to be measured of the normal collagen fibrils there were no significant differences in the depth of D-periods between any of the three groups: AngII Group 1 (3.3 ± 0.4), AngII Group 2 (3.3 ± 0.6 nm), and saline (3.3 ± 0.7 nm). However, the abnormal collagen fibrils showed a

significantly reduced D-depth which was often not measurable. A summary of these results can be found in Table 2.

Table 2: Summary of measurements from collagen fibrils in murine adventitia.

	Length of D-Period (nm)	Depth of D-Period (nm)
DDR1 KO	67.5 ± 3.8 (8 mice, 279 periods)	3.6 ± 0.6 (8 mice, 276 periods)
WT	67.0 ± 1.8 (8 mice, 253 periods)	3.0 ± 0.4 (8 mice, 251 periods)
AngII, Group 1	62.8 ± 2.6 (3 mice, 9 periods)	3.3 ± 0.4 (3 mice, 9 periods)
AngII, Group 2	67.0 ± 3.5 (2 mice, 6 periods)	3.3 ± 0.6 (2 mice, 6 periods)
Saline	67.2 ± 4.4 (4 mice, 20 periods)	3.3 ± 0.7 (4 mice, 20 periods)
In-Vitro Collagen	66.2 ± 10.2 (24 periods)	1.6 ± 0.4 (24 periods)
In-Vitro Collagen + DDR1-Fc	No Discernible Banded Structure	No Discernible Banded Structure

Discussion

Deposition of collagen has been shown to be affected by the presence of collagen binding proteins (CBP) during fibril formation, including DDR1 (Franco et al., 2008). In addition, our laboratory has shown how collagen fibril structure in-vitro is affected by the presence of DDR1 (Agarwal, 2016). A major goal of this study was to quantify any changes in the structure of collagen in the presence vs. absence of DDR1 in-vivo. Atomic force microscopy of the adventitial layers of murine aorta showed that the depth of the D-period gap in DDR1 KO mice is significantly greater than that of WT mice. However, the length of the D-period shows no significant difference between these two groups of mice. The differences in the depth of the gap point to the fact that the fibrils may not be formed in the same fashion when DDR1 is absent.

The ultrastructure of collagen fibrils may also be altered in models that display pathologies related to collagen deposition. One such pathology is abdominal aortic aneurysm (AAA). AAA is characterized by remodeling of collagen in the abdominal aorta following a disruption of the normal function of the tunica media and adventitia. The ultrastructure features of the remodeled collagen in AAA have yet to be quantified (Hans et al., 2012). Changes in the ultrastructure during AAA may contribute to future complications of the condition such as expansion or rupture of the aneurysm.

ApoE KO mice infused with AngII represents a standard model for abdominal aortic aneurysm. This model is able to present the typical characteristics of AAA and can be compared to ApoE KO mice infused with saline instead of AngII as a control. The AFM images of the AngII

and saline-infused both show abnormal formation of collagen fibrils in some regions and normal fibril structure in other regions. The data was acquired from imaging showed no significant differences in the length and depth of D-period in any of the three groups. However, the depth of D-periods was significantly compromised in abnormal fibrils, similar to the feature observed in in-vitro reconstituted collagen fibrils in the presence of DDR1. Other imaging modalities such as TEM have confirmed our AFM observation on presence of abnormal fibrils in AAA (Tonniges et al., 2016c).

Taken together our results indicate that whereas the length of D-period remains preserved in collagen fibrils, the depth of D-periods can undergo significant changes as demonstrated in earlier studies of the cornea (Table 1) as well as our present studies on adventitial collagen (Table 2). Characterizing the depth of D-periods in collagen fibrils can thus serve as a novel parameter in quantifying changes in the collagen fibril structure. Change in the collagen fibril structure may affect the exposure of amino acid sequences that are not normally available on the surface of the collagen fibrils, causing altered interactions with other ECM proteins. Changes in the structure may also affect the mechanical properties of collagen. The study of the collagen structure can provide novel insights into the pathogenesis of vascular diseases.

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