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Quantification of aortic and cutaneous elastin and collagen morphology in Marfan syndrome by multiphoton microscopy

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ABSTRACT

In a mouse model of Marfan syndrome, conventional Verhoeff-Van Gieson staining displays severe fragmentation, disorganization and loss of the aortic elastic fiber integrity. However, this method involves chemical fixatives and staining, which may alter the native morphology of elastin and collagen. Thus far, quantitative analysis of fiber damage in aorta and skin in Marfan syndrome has not yet been explored. In this study, we have used an advanced noninvasive and label-free imaging technique, multiphoton microscopy to quantify fiber fragmentation, disorganization, and total volumetric density of aortic and cutaneous elastin and collagen in a mouse model of Marfan syndrome. Aorta and skin samples were harvested from Marfan and control mice aged 3-, 6- and 9-month. Elastin and collagen were identified based on two-photon excitation fluorescence and second-harmonic-generation signals, respectively, without exogenous label. Measurement of fiber length indicated significant fragmentation in Marfan vs. control. Fast Fourier transform algorithm analysis demonstrated markedly lower fiber organization in Marfan mice. Significantly reduced volumetric density of elastin and collagen and thinner skin dermis were observed in Marfan mice. Cutaneous content of elastic fibers and thickness of dermis in 3-month Marfan resembled those in the oldest control mice. Our findings of early signs of fiber degradation and thinning of skin dermis support the potential development of a novel non-invasive approach for early diagnosis of Marfan syndrome.

1. Introduction

Marfan syndrome (MFS) is an autosomal dominant disorder of connective tissue caused by mutations in fibrillin-1 (*FBN1*) affecting multiple systems, including cardiovascular, skeletal, ocular and pulmonary (Dietz et al., 1991; Pyeritz, 2000). *FBN1* is a large extracellular matrix (ECM) glycoprotein and a major component of the *FBN1*-rich microfibrils, which provide a scaffold that is crucial during formation and maturation of elastic fibers (Kielty et al., 2005). The primary load-bearing components of the aortic wall are elastic fibers, collagen fibrils, and smooth muscle cells (SMCs). Degeneration of elastic fibers and alterations in collagen diminish the load-bearing capacity of the aorta in MFS and promote microdissection of the aorta. Consequently, mortality in MFS is usually due to progressive thoracic aortic aneurysm (TAA), dissection, and rupture (Judge and Dietz, 2005). TAA is characterized by destruction of elastin and collagen in the media and adventitia, transmural infiltration of lymphocytes and macrophages, loss of medial SMCs with thinning of the vessel wall, which lead to impairment of vascular cell function, and deterioration of aortic mechanical properties (Ailawadi et al., 2003). Our group has previously demonstrated that progression of TAA in MFS is associated with up-regulation of matrix metalloproteinase (MMP)-2 and -9, the endopeptidases that cleave most of the constituents of ECM such as microfibrils. Increased MMP activity is accompanied by extensive degeneration of elastic fibers and collagen, resulting in loss of ECM integrity, endothelial dysfunction, and reduction of smooth muscle contractility (Chung et al., 2007a,b, 2008a; Yang et al., 2010).

Skin is the largest organ of the body based on surface area. Like the aorta, elastin and collagen are two major ECM components of the skin dermis (Chen et al., 2011). Elastin provides the majority of the resilience and elastic properties of skin, whereas collagen gives the skin strength, texture, durability, mechanical and struc-

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tural integrity (Mochizuki et al., 2002). Previous studies have shown that various proteases, including MMPs, are responsible for the degradation of elastin and collagen in skin (Brenneisen et al., 1997, 1996). Thus far, quantitative analyses of elastic fiber damage and breakdown of collagen in MFS skin have not been explored. Existing studies are based on histological method, electron microscopy, and immunohistochemical techniques (Amadeu et al., 2004; Godfrey et al., 1990). These methods usually involve fixation, staining, and dehydration of biopsied tissue specimen, which alter the native morphology of elastin and collagen. Recently, multiphoton microscopy (MPM), where excitation of fluorescent probes occurs through the simultaneous absorption of two or more photons of longer wavelengths, in the near-infrared regions (Denk et al., 1990; Zipfel et al., 2003), has been employed to yield three-dimensional (3-D) rendering of the structures of elastin and collagen in living tissue up to a very high depth.

Because MPM is able to non-invasively reveal the ultra-structure of elastin and collagen (Abraham et al., 2011), this technique has gained considerable favor in cardiovascular research and dermatology (Chen et al., 2011; Lin et al., 2007). Its main advantage is the capability of observing non-fixed, unstained tissue samples (Abraham et al., 2011). with the potential of performing non-invasive *in vivo* measurements directly on a patient (Masters et al., 1997). The aorta and skin contain naturally occurring fluorophores that can be imaged using MPM without the need for exogenous contrast agents. These include elastin, collagen, melanin, keratins, porphyrins, NAD(P)H and flavins (Levitt et al., 2011). Collagen in the aorta and dermis of the skin produces a second harmonic generation (SHG) signal, which can be differentiated from two-photon excited fluorescence (TPF) generated by elastin (Tang et al., 2006).

The aim of this study is to use MPM for assessing the progress of elastin and collagen damage in the large arteries and skin dermis of a mouse model of MFS. The morphology and total volumes of elastin and collagen measured using MPM in MFS mice aorta and skins are compared with control mice in different age groups. The novel aspect of this study is the non-invasive recording and comparison of defects in elastin and collagen present in aorta and the inner layer of the skin dermis; with the long-term aim of developing an *in vivo* skin test for early non-invasive diagnosis of MFS and other connective tissue disorders.

2. Materials and methods

2.1. Experimental animals

All experiments were performed using a previously described transgenic mouse models, harboring an *Fbn1* allele encoding mutation C1039G (a cysteine substitution $Cys^{1039} \rightarrow Gly$), in an epidermal growth factor-like domain of fibrillin-1 ($Fbn1^{C1039G/+}$) (Chung et al., 2007a,c, 2008b; Judge et al., 2004; Yang et al., 2010). Heterozygous mice were bred with wild-type mice (C57BL/6) to generate control ($Fbn1^{+/+}$) and MFS ($Fbn1^{C1039G/+}$) mice, which were housed in the institutional animal facility. All animal procedures were approved by the institutional animal ethics board [reference number A11-0018], and all animals received humane care in compliance with the Guide for the Care and Use of Laboratory Animals (www.nap.edu/catalog/5140.html). Mice were anaesthetized by inhalation of 3% of isoflurane (Baxter Corporation, Mississauga, Canada), and the adequacy of anesthesia was confirmed by pedal reflex. Mice were then sacrificed by cervical dislocation.

2.2. Aorta tissues preparation for histologic and multiphoton imaging

The thoracic aorta (~10–14 mm) was dissected from control and MFS mice, at the age of 3-, 6-, and 9-month (3-month: control $n = 5$, MFS $n = 4$; 6-month: control $n = 5$, MFS $n = 4$; 9-month: control $n = 3$, MFS, $n = 4$). Specimens were washed in cold PBS (pH = 7.4) and cut into segments for use in MPM and histologic analysis. The ascending aortic root was transected above the level of the aortic valve, and 2–3 mm transverse sections were cut to two or three pieces (length, 1 mm/each). The curving aortic arch (length, ~3 mm) and the descending aorta (length, 3–5 mm) were cut to 3 or 4 pieces, respectively (1 mm/each piece). The aortic tube segments were then mounted vertically on a petri dish, after which they were washed and immersed in PBS for multiphoton imaging.

2.3. Skin tissues preparation for multiphoton imaging

The skin from the dorsal surface was dissected from control and MFS mice (3-, 6- and 9-month old) after the hairs were shaved and removed by applying hair removal, followed by washing in cold PBS. The harvested skin specimens were flattened and cut into two halves. One half was embedded in optimum cutting temperature compound (VWR, West Chester, PA), snap frozen in liquid nitrogen, and cut into transverse sections at 50 μm thick for multiphoton imaging. The other half was utilized for histochemistry.

2.4. Features of the MPM system and experimental set-up

The basic outline of a MPM system capable of both TPF and SHG signal detections is presented in Fig. 1 and was described in details elsewhere (Abraham et al., 2011). In our present study, SHG signal originating from collagen was obtained from the emission wavelength at 440 nm, which only arises at half of the excitation wavelength 880 nm. The TPF signal originating from elastin was also obtained from the excitation wavelength at 880 nm, attributing to the measured broadband emission spectrum ranging from 400 to 650 nm with a peak at 500 nm, as previously described (Abraham and Hogg, 2010).

2.5. Histological staining of aortas and skin

Three parts of aorta and the second half of dorsal skin samples were fixed in 10% buffered formalin for 48 h, after which they were immersed in 70% ethanol overnight at 4 °C, and embedded in paraffin. Specimens were cut into 5 μm thick cross-sections. Tissue sections were deparaffinized in xylene and rehydrated in graded ethanol. Elastic fibers of aortas and skins were stained by use of Accustain® Elastic Stain kit (Sigma–Aldrich, St. Louis, MO) according to the manufacturer's standard procedure. Briefly, rehydrated tissue sections were placed in a Coplin jar containing working elastic stain solution for 10 min. Sections were rinsed in deionized water and then differentiated in working ferric chloride solution for 30–60 s. Differentiation was stopped with several changes of tap water. Sections were then rinsed in 95% alcohol to remove iodine and then in Van Gieson solution for 3–5 min (Lillie, 1965). Following the staining procedure, samples were dehydrated through graded ethanol and xylene, and mounted with mounting medium with coverslip.

2.6. Quantitative and statistical analysis

3-D reconstruction of aortic segments and skin, as well as the quantification of volumetric density of elastin and collagen distinguished by TPF and SHG signals, respectively, was performed using Volocity® 6.1.1 image-processing software (PerkinElmer, Waltham, MA) as previously descried (Suzuki et al., 2012). The elastic fiber fragmentation was determined using Image-Pro-Plus 6.0 software package (Media Cybernetics, Bethesda, MA), by tracing elastin and measuring their lengths in pixels followed by unit conversion to micrometer as previously described (Zhou et al., 2012).

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