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Lysyl oxidase-mediated collagen crosslinks may be assessed as markers of functional properties of tendon tissue formation

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ABSTRACT

Mechanical property elaboration of engineered tissues is often assumed on the basis of gene and protein characterizations, rather than mechanical testing. However, we recently demonstrated that mechanical properties are not consistently correlated with matrix content and organization during embryonic tissue development. Based on this, mechanical properties should be assessed independently during natural or engineered tissue formation. Unfortunately, mechanical testing is destructive, and thus alternative means of assessing these properties are desirable. In this study, we examined lysyl oxidase (LOX)-mediated crosslinks as markers for mechanical properties during embryonic tendon formation and the potential to detect them non-destructively. We used tandem mass spectrometry (LC-MS/MS) to quantify changes in hydroxylysyl pyridinoline (HP) and lysyl pyridinoline (LP) crosslink density in embryonic chick tendon as a function of developmental stage. In addition, we assessed a multiphoton imaging approach that exploits the natural fluorescence of HP and LP. With both techniques, we quantified crosslink density in normal and LOX-inhibited tendons, and correlated measurements with mechanical properties. HP and LP crosslink density varied as a function of developmental stage, with HP-to-dry mass ratio correlating highly to elastic modulus, even when enzymatic crosslink formation was inhibited. Multiphoton optical imaging corroborated LC-MS/MS data, identifying significant reductions in crosslink density from LOX inhibition. Taken together, crosslink density may be useful as a marker of tissue mechanical properties that could be assessed with imaging non-destructively and perhaps non-invasively. These outcomes could have significant scientific and clinical implications, enabling continuous and long-term monitoring of mechanical properties of collagen-crosslinked tissues or engineered constructs.

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1. Introduction

Functional properties of engineered tissues are typically assessed at late time-points, often due to limited sample numbers and difficulty handling fragile tissue constructs prior to significant extracellular matrix (ECM) protein deposition. Thus, assessments of tissue formation, especially at earlier stages, are frequently based on protein production and organization. However, proper elaboration of mechanical properties is critical for tissues with mechanically demanding roles in the body. Tendons transfer load from muscle to bone to enable movement and locomotion, and thus their mechanical properties are essential for normal function. Unfortunately, adult tendon fails to heal when injured, or heals with scar tissue that possesses aberrant mechanical properties even after surgical repair, compromising physical function and leading to secondary disorders such as arthritis [1,2]. Aberrant mechanical properties of scar tissue are associated with abnormal matrix content and organization. Consequently, efforts to enhance healing or regeneration have focused on controlling protein expression and tissue microstructure. Similarly, during engineered or native tissue formation, the elaboration of mechanical properties and structural integrity are commonly assumed if increased ECM content and organization are observed. Thus, in multiple contexts, researchers often forgo mechanical property characterization and instead rely on tissue morphology and biochemical content to assess functional tissue formation.

Collagen crosslinking has been reported to contribute significantly to the mechanical properties of adult homeostatic tendon, as well as to those of dysfunctional tendon that develop with aberrant, disorganized matrix during healing, disease progression (e.g., tendinopathy) and aging [1–3]. We recently showed that inhibition of lysyl oxidase (LOX) activity to reduce collagen crosslinking during embryonic chick tendon development resulted in dramatic decreases in nanoscale elastic modulus [4]. Unexpectedly, we also

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found that these changes in mechanical properties with enzymatic crosslinking inhibition did not affect total collagen or glycosaminoglycan (GAG) content, or apparent matrix organization [4]. The lack of correlation between mechanical property changes and ECM morphology and content was a significant finding, demonstrating the need to also characterize functional properties of newly forming tissues. Unfortunately, mechanical testing is time-consuming and destructive, requiring high sample numbers and precluding the ability to continuously monitor a single sample over time. Therefore, non-destructive means to monitor functional tissue formation would be desirable. Based on our previous study, we proposed that enzymatic crosslink density correlates directly with mechanical properties during development, and that LOX-mediated crosslinks may be useful as functional markers for tissue formation. The objectives of this study were to characterize LOX-mediated crosslink densities of developing embryonic tendon, to assess the relationship between enzymatic crosslink density and tendon mechanical properties during tissue formation and to determine whether these crosslinks may be detected non-destructively with imaging.

Collagen crosslinks in tendon include GAGs, non-enzymatic and enzymatic (LOX-mediated) crosslinks. GAGs appear to physically bridge adjacent collagen fibrils in tendon. However, mechanical testing of proteoglycan-deficient tendons or those digested to remove GAGs found decreases in elastic modulus in some tendons [5] and increases or no change in others [6]. In embryonic tendons, we previously demonstrated no correlation between total GAGs and elastic modulus [4]. Other crosslinks implicated in tendon mechanical properties include non-enzymatic collagen crosslinks seen in aging, such as pentosidine [2], though incubation of tendons with glucose to generate pentosidine produced minimal mechanical effects [7]. Taken together, non-enzymatic crosslinks may not be significant contributors to developing tendon mechanical properties. Therefore, we focused on LOX-mediated crosslinks.

We recently demonstrated that LOX crosslinks contribute to mechanical properties of embryonic tendon [4], but did not quantitatively characterize their relationship with mechanical properties of developing tissue. The few published studies focused on LOX-mediated collagen crosslinking in developing tendon are limited to a single embryonic time-point [8]. In contrast, LOX-mediated crosslinking has been studied extensively in adult tendon. LOX oxidatively deaminates specific telopeptidyl lysine and hydroxylysine residues of the collagen molecule [9] to facilitate the formation of a covalent bond between three laterally aligned collagen molecules [10]. The most prevalent enzymatic crosslink in adult tendon appears to be the trivalent hydroxylysyl pyridinoline (HP) crosslink, and to a lesser degree the trivalent lysyl pyridinoline (LP) crosslink [10,11]. Interestingly, these crosslinks seem to play distinct roles in events primarily outside of homeostasis. In normal tissues, the HP-to-collagen ratio was positively correlated to elastic modulus of tendons in goat [12], but not in human [13] or equine [14]. In human, normal tendons maintained constant levels of HP- and LP-to-collagen ratios in the adult [2], whereas aging tendons showed increased HP-to-collagen ratio, higher elastic modulus and significantly lower collagen content than younger tendons [3]. Chronically injured adult human tendons showed significantly higher HP- and LP-to-collagen ratios compared to non-degenerated tendons [2]. Additionally, acutely injured rabbit ligaments sharply reduced the HP-to-collagen ratio, which progressively increased over a 40-week healing period along with bulk elastic modulus [1]. Taken together, the lack of correlation between modulus and enzymatic crosslinks during homeostasis, but apparent correlation with changes with ageing and injury, suggest roles for LOX-mediated crosslinking in events that affect mechanical properties, such as tendon formation, degeneration, injury and healing. Based on this, we proposed that enzymatic crosslink density changes during embryonic development, prior to the formation of additional non-enzymatic (e.g., pentosidine) crosslinks or the introduction of confounding environmental factors, may correlate with mechanical property elaboration.

Traditional methods to measure crosslink density include high-performance liquid chromatography (HPLC) and mass spectrometry techniques. Both methods require tissue hydrolysis to solubilize crosslinks for analysis. Drawbacks to hydrolysis are that the sample is destroyed during measurement and it is not possible to resolve spatial differences in crosslink density at small scales. In contrast, non-destructive and potentially non-invasive techniques could enable repeated measurements of crosslink density in a single sample over a length of time, or allow for additional assays subsequent to crosslink measurements. This would be advantageous for in vitro and in vivo applications where samples are limited (e.g., small, rare, expensive) or too important to be destroyed. Infrared microspectroscopy has been utilized to assess divalent crosslinks in bone [15], though a recent report concluded that this method is actually sensitive to the secondary structure of collagen instead of collagen crosslinking [16]. Therefore, there is a need for a specific and non-destructive method to quantify collagen crosslink density in tendon and other collagenous tissues.

In this study, we aimed to provide a quantitative profile of LOX-mediated crosslinks during tendon development and to examine the relationship between elastic modulus and enzymatic collagen crosslink density in embryonic tendon. We hypothesized that LOX-mediated crosslink density increases during embryonic development, and that crosslink density is directly correlated with nanoscale elastic modulus. We employed tandem mass spectrometry (LC-MS/MS) to characterize changes in HP and LP density and collagen content in embryonic tendon as a function of developmental stage and LOX activity inhibition. To assess a non-destructive method to characterize enzymatic crosslink density, we also utilized multiphoton microscopy to optically measure crosslink density and collagen content of normal and LOX inhibitor-treated embryonic tendons, by exploiting the natural fluorescence of HP and LP crosslinks and second harmonic generation (SHG) signal from fibrillar collagen. With these analyses, we profiled HP and LP crosslink density during tendon development and examined potential relationships between HP and LP crosslinks with elastic moduli to characterize whether collagen crosslink density may be a functional marker of developing tendon tissue. Characterization of these crosslinks and understanding their relationship with mechanical properties could provide functional markers of tendon formation, which would be useful for assessing quality of tissue regeneration during healing or engineered tissue development.

2. Materials and methods

2.1. In ovo culture and tendon harvest

All animal procedures received prior approval from the university institutional animal care and use committee board. All reagents were from Sigma-Aldrich Co. (St Louis, MO) unless otherwise specified. White leghorn chick embryos (University of Connecticut Poultry Farm, Storrs, CT) were cultured in a humidified rocking incubator at 37.5 °C. Embryos were sacrificed and staged according to Hamburger and Hamilton (HH) [17] at HH 28, 35, 40 and 43, equivalent to approximately days 5.5, 9, 14 and 18 out of a 20-day gestation period, respectively. At 24 h before each time-point, embryos were injected with 200 μl of β-aminopropionitrile (BAPN; inhibitor of LOX activity) in saline equivalent to either 0, 5 or 15 mg g^{-1} of dry embryo mass [18] into the chorioallantoic membrane [19]. The shell hole was sealed with liquid paraffin and embryos were cultured in ovo for an additional 24 h.

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