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Collagen fibril morphology and organization: Implications for force transmission in ligament and tendon

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Abstract

Connective tissue mechanical behavior is primarily determined by the composition and organization of collagen. In ligaments and tendons, type I collagen is the principal structural element of the extracellular matrix, which acts to transmit force between bones or bone and muscle, respectively. Therefore, characterization of collagen fibril morphology and organization in fetal and skeletally mature animals is essential to understanding how tissues develop and obtain their mechanical attributes. In this study, tendons and ligaments from fetal rat, bovine, and feline, and mature rat were examined with scanning electron microscopy. At early fetal developmental stages, collagen fibrils show fibril overlap and interweaving, apparent fibril ends, and numerous bifurcating/fusing fibrils. Late in fetal development, collagen fibril ends are still present and fibril bundles (fibers) are clearly visible. Examination of collagen fibrils from skeletally mature tissues, reveals highly organized regions but still include fibril interweaving, and regions that are more randomly organized. Fibril bifurcations/fusions are still present in mature tissues but are less numerous than in fetal tissue. To address the continuity of fibrils in mature tissues, fibrils were examined in individual micrographs and consecutive overlaid micrographs. Extensive microscopic analysis of mature tendons and ligaments detected no fibril ends. These data strongly suggest that fibrils in mature ligament and tendon are either continuous or functionally continuous. Based upon this information and published data, we conclude that force within these tissues is directly transferred through collagen fibrils and not through an interfibrillar coupling, such as a proteoglycan bridge.

Keywords: Extracellular matrix; Structure-function; Scanning electron microscopy; Three-dimensional matrix organization; Cell–matrix interaction

1. Introduction

Collagens, the primary structural elements of the extracellular matrix, are the most abundant proteins in tissues such as ligament, tendon, cartilage, bone, cornea, and skin. Type I collagen assembles, via collagen molecules, into collagen fibrils which are long filamentous structures which aggregate to form collagen fibers (Nimni and Harkness, 1988). In vivo, type I collagen fibrillogenesis is a multi-step process involving intracellular and extracellular compartments defined by the fibroblast (Birk and Trelstad, 1984, 1986; Birk et al., 1989; Canty et al., 2004). Collagen fibril segments then form intermediate structures that assemble into collagen fibrils and undergo post-depositional growth during embryonic development (Birk et al., 1995, 1989, 1997). For instance, in tendons from embryonic chickens, fibrils substantially increase in length between 14 and 17 days (Birk et al., 1995). After 17 days of embryonic development, fibril length dramatically increases (Birk et al., 1996, 1995). By 18 days of embryonic development, Birk et al. had great difficulty identifying both ends of the collagen fibrils when examining the tendon over the same tissue length in which both ends of fibrils were easily identifiable in 14 day embryos; further illustrating the rapid and substantial fibril lengthening at this stage of development (Birk et al., 1997). This increase in fibril length during embryonic development may be the result of lateral association or fusion between collagen fibrils, producing longer and larger diameter fibrils (Birk et al., 1997), or by tip-to-tip fusions of collagen fibrils to produce longer fibrils (Graham et al., 2000), or both. Exact mechanisms of fibril lengthening during development require further study.

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During collagen fibrillogenesis, proteoglycans play a large role in guiding and stabilizing collagen fibril formation and maturation. Several studies have shown that decorin, lumican, and fibromodulin, members of the small leucine-rich proteoglycan (SLRP) family, play a role in regulating collagen fibril organization and maturation (Birk et al., 1995; Chakravarti et al., 1998; Danielson et al., 1997; Ezura et al., 2000; Graham et al., 2000; Jepsen et al., 2002; Keene et al., 2000; Scott, 1996; Scott et al., 1981; Svensson et al., 1999; Vogel and Trotter, 1987). In relation to type I collagen, decorin is located along the fibril shaft and is attached to the fibril surface via noncovalent bonding (Scott and Orford, 1981) with its glycosaminoglycan (GAG) chain extending laterally from the fibril, possibly maintaining hydration and interfibrillar spacing (Scott, 1988), and is absent at collagen fibril ends where tip-to-tip fusion can occur (Graham et al., 2000). In addition to decorin, both fibromodulin and lumican have also been implicated in regulating collagen fibrillogenesis. Like decorin, these SLRPs are envisioned as having a horseshoe shaped core protein in which the concave surface laterally associates with collagen and the GAG chain extends laterally away from the fibril (Scott, 1996; Weber et al., 1996). When collagen fibrils from mice deficient in fibromodulin and/or lumican are examined, they exhibit abnormal development in terms of diameter and shape (Chakravarti et al., 1998; Ezura et al., 2000; Jepsen et al., 2002; Keene et al., 2000; Svensson et al., 1999). Therefore, in vivo SLRPs, such as decorin, fibromodulin, and lumican, strongly regulate multiple aspects of collagen fibrillogenesis and consequent integrity.

As described above, during embryonic development collagen fibrils are known to be discontinuous units, which profoundly increase in length as development progresses. Less information is known about the length of the collagen fibril at birth, through the skeletally immature growth phase, and after skeletal maturity has been reached. It is known that material properties, such as ultimate stress, of the collagenous extracellular matrix (ECM) increase more than three fold from skeletally immature animals to more developed or skeletally mature animals (Beredjiklian et al., 2003; McBride et al., 1988; Provenzano et al., 2002a; Woo et al., 1986), implying a substantial change to the extracellular matrix must occur as the animal matures; as indicated via continuous collagen fibers in chick tendon during late embryonic development and shortly after birth (McBride et al., 1985). To explain these changes, in addition to known changes associated with cross-linking and fiber morphology, experiments have been conducted and hypotheses discussed regarding the length of collagen fibrils within collagen fibers in mature animals, and the organization and composition of the ECM, particularly the proteoglycan–collagen interaction, in skeletally mature animals. One such study by Craig et al. (1989) theoretically illustrates that collagen fibril length increases from birth to maturity with mature fibrils reaching lengths greater than 10 mm. Yet, controversy still exists regarding the length and continuity of collagen fibrils in skeletally mature tendons and ligaments.

Many authors assume or conclude that the strong majority of collagen fibrils are long (millimeters in length) and either span the length of the ligament or tendon or are long enough to be considered functionally continuous (Birk et al., 1995, 1997; Craig et al., 1989; Graham et al., 2000; Holmes et al., 1998; Parry and Craig, 1984). For instance, utilizing transmission electron microscope to examine fibril length in mature tendon, Trotter and Wofsy (1989) examined 5639 tendon fibrils (over 4.26 mm of fibril length) and found two ends from small fibrils, while Parry and Craig (1984) examined 1000 fibrils and Craig et al. (1989) 1368 fibrils and found no ends. In contrast, other authors suggest that the majority of fibrils in mature mammalian tendon and/or ligament are short discontinuous fibrils (Caprise et al., 2001; Dahners et al., 2000; Derwin and Soslowsky, 1999; Derwin et al., 2001; Mosler et al., 1985; Nemetschek et al., 1983; Raspanti et al., 2002; Redaelli et al., 2003; Robinson et al., 2004). Accompanying the hypothesis of short discontinuous fibrils is the hypothesis that force must be transferred between fibrils through a mechanical coupling (Mosler et al., 1985; Nemetschek et al., 1983), typically SLRPs (Caprise et al., 2001; Dahners et al., 2000; Derwin and Soslowsky, 1999; Derwin et al., 2001, Redaelli et al., 2003; Robinson et al., 2004). Although fibril length was not addressed, in mammalian tendon, Cribb and Scott (1995) proposed the concept of proteoglycan bridges playing a role in transmitting and resisting tensile stress. More recent data, however, have not strongly supported the concept of a force transmitting proteoglycan bridge. Dahners et al. (2000) hypothesized that "decorin–fibronectin binding is an important link in interfibrillar bonding" and applied NKISK (an agent that inhibits binding of decorin to fibronectin) to isolate intact fibrils from mature rat ligament and tendon. Only ten intact small diameter fibrils ligament and sixteen possibly intact small diameter tendonous fibrils were obtained. That is, the vast majority of fibrils were either long or not isolated by this method, and administration of NKISK had no detrimental effect on tissue strength or stiffness (Caprise et al., 2001). Furthermore, work in mov13 mice, reveals no significant difference in maximum load, stress, stiffness, modulus, or total collagen between tendon fascicles from mov13 and control mice, even though the ratio of decorin to collagen is decreased in the mov13 mice (Derwin and Soslowsky, 1999; Derwin et al., 2001). In accordance with the above studies, tendons from decorin knockout mice reveal no significant difference in maximum load, stress, stiffness, or modulus between age matched controls (Forslund et al., 2002, Gimbel et al., 2002, Lin et al., 2002, Robinson et al., 2004) and the adding of decorin to self-assembled collagen fibers does not significantly increase the ultimate stress of the tissue, but may help facilitate slippage between fibrils (Pins et al., 1997). Additionally, mice deficient in both fibromodulin and lumican show an appreciable decrease in the modulus (Jepsen et al., 2002). Yet, due to the extremely abnormal cross-sectional morphology and distribution of collagen fibrils, resulting from the combined lumican/fibromodulin deficiency during development, it would appear that changes in collagen cause the

reduced mechanical properties in these mice (Jepsen et al., 2002). Lastly, Redaelli et al. (2003) used a theoretical model to investigate the concept of proteoglycan bridges to transmit load through a matrix of short discontinuous fibrils. However, this model was unable to achieve the properties of mature tissue; and only approached the properties of mature tissue when the model's maximum fibril length was utilized. Furthermore the model had a fixed transverse distance between fibrils with a GAG length and interfibrillar distance maximized to a level that is far beyond the transverse distance between fibrils in ligamentous or tendinous tissue (e.g. (Frank et al., 1992)). This was required in order to accommodate GAG chain strain of over 800% so that the model would bear substantial loads. Thus, the proteoglycan bridge model was unable to predict mature tissue behavior and contained assumptions that challenge the fidelity of the model. In summary, these studies indicate that SLRPs play a strong role in collagen fibrillogenesis, but do not support the hypothesis of SLRPs forming an interfibrillar mechanical coupling for force transfer. Therefore, in order to understand the mechanisms by which collagenous connective tissues form such remarkable structural elements, the length and organization of collagen fibrils in mature tissue are in need of further study.

Given that few fibril ends have been reported in mature ligament or tendon in situ, few intact fibrils have been extracted from mature ligament or tendon in vitro, and current experimental biomechanical studies do not support the hypothesis of force transfer from discontinuous fibrils through a proteoglycan bridge; the discontinuous fibril theory currently lacks strong support. Alternatively, fibril continuity may

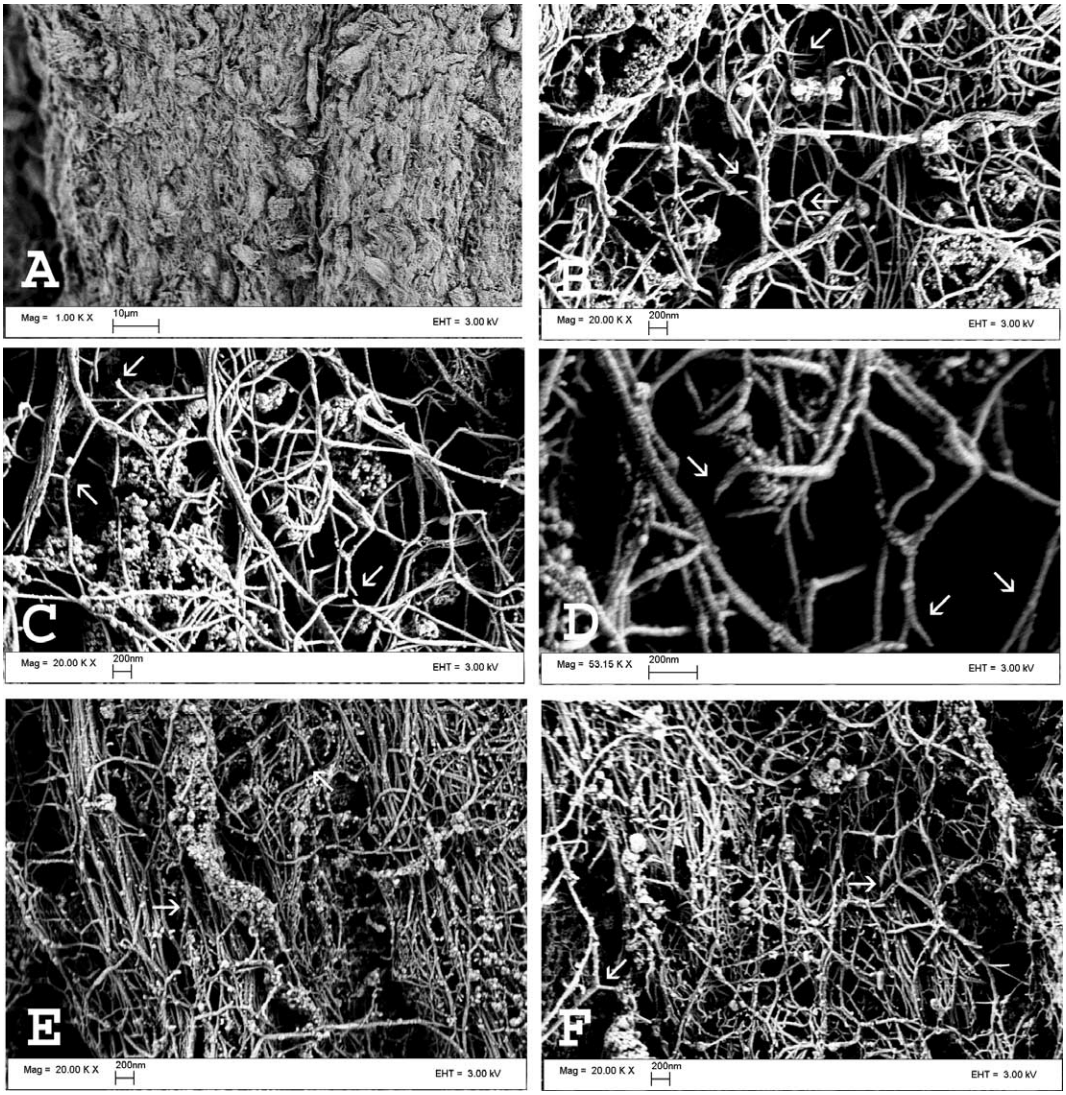

Fig. 1. Collagen fibril morphology during early development. Organization and structure of collagen fibrils from bovine tendon and ligament during early fetal development (bovine day F40; standard gestation >280 days). At this stage in development, collagen fibril ends are clearly imaged with SEM. Additionally, tendon and ligament fiber and fibril morphology display similar characteristics with the collagen fibers partially organized, with a trend toward axial alignment (A: fetal bovine tendon). At higher magnifications (B–D**:** tendon; E–F: medial collateral ligament) the fibrils show substantial interweaving, tapered and rounded collagen fibril ends are clearly visible (see arrows in D) and fibril bifurcations/fusions are present (examples of ends and bifurcating/fusing fibril are indicated with arrows). Note: the presence of (non-damaged) tapered and more rounded collagen fibril ends that are clearly displayed with SEM and consistent with previous reports using alternative techniques (Holmes et al., 1992, 1998).

provide the load transfer mechanism through these tissues. This study therefore examines collagen fibrils in fetal and mature ligament and tendons to examine their length, continuity, and organization. Scanning electron microscopy (SEM) was chosen for imaging since other methods such as second harmonic generation and optical coherence tomography do not provide high enough resolution (nm scale) to distinguish the fibril characteristics of interest in dense collagen bundles, and transmission electron microscopy, while providing detailed information about collagen morphology, is limited by its planar images. In this study, the ability of SEM to detect collagen fibril ends was validated in ligaments and tendons from fetal

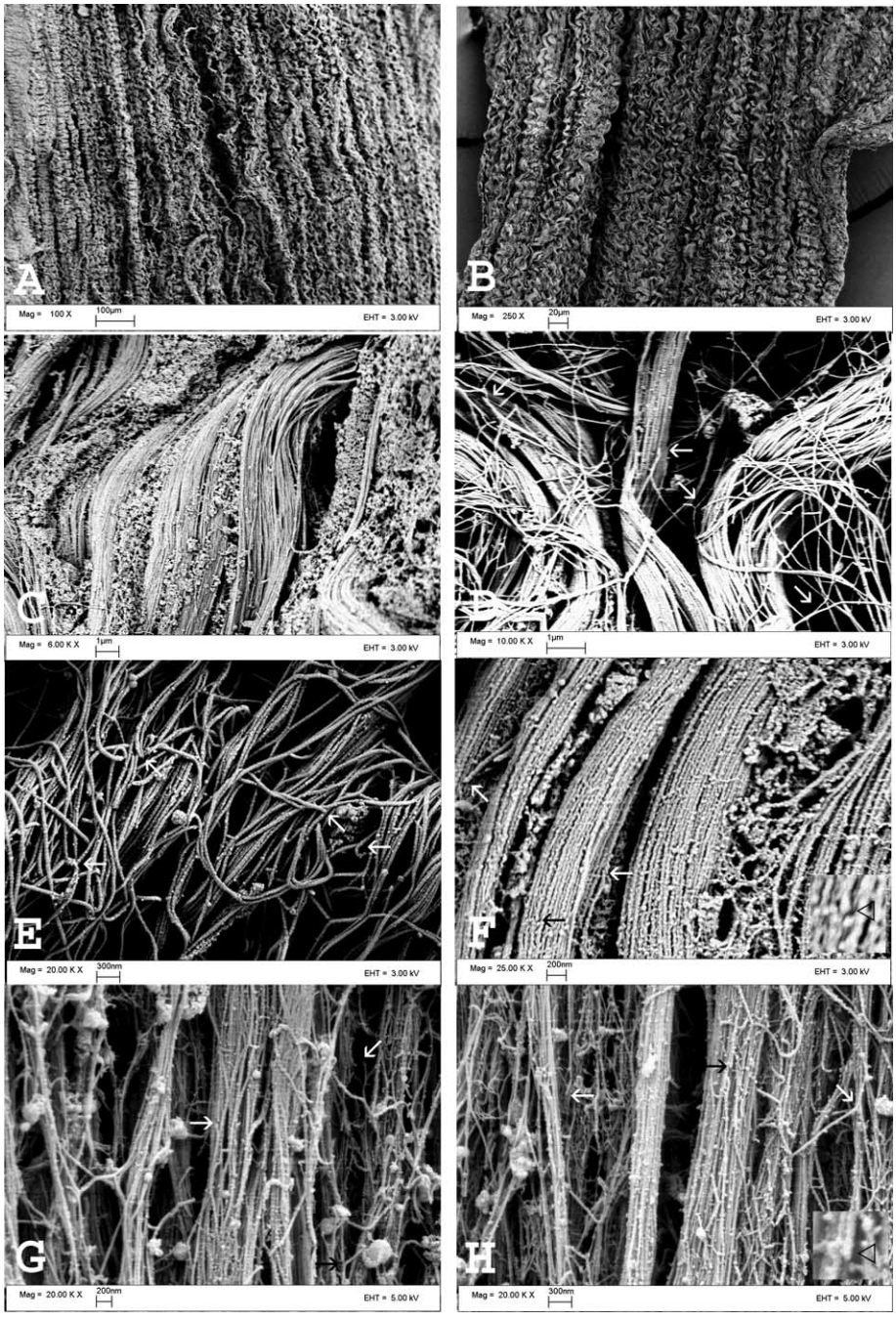

Fig. 2. Collagen fibril morphology during late development. Collagenous matrix organization and fibril morphology in feline (day F50; standard gestation ~60 days) and rat (day F18; standard gestation ~21 days) tendon and ligament during the late fetal development stage. At this later stage in development, collagen fibril ends are clearly imaged with SEM. Additionally, examination of the tissues at low magnification (A–B), clearly demonstrates formation of fibril bundles (fibers) that possess the characteristic crimp morphology seen in mature tendon and ligament (A: patella tendon, B: medial collateral ligament). At higher magnification (E–H), feline patella tendons (C,D,F) and medial collateral ligaments (E), and rat medial collateral ligaments (G) and patella tendons (H) contain collagen fibril ends, including tapered and rounded fibril ends (indicated with arrows along with fibril bifurcations/fusions) and ends contained within dense fibril bundles are clearly visible (black arrows in F and H; open arrow heads in enlarged cut outs). These data, along with data illustrated in Fig. 1, clearly validate the ability of SEM to image collagen fibril ends in tissue of differing size and to distinguish fundamental collagen fibril morphology.

animals of differing sizes; and fibril morphology in ligaments and tendons from skeletally mature animals (a system in which the collagen fibril morphology has received less study than that performed in fetal tissues) was extensively imaged to elucidate the presence of fibril ends and examine fibril organization and continuity. Additionally these findings, combined with previously published data, are discussed in relation to force transmission in these tissues.

2. Results

Fibril ends have been observed in embryonic tissues (e.g. (Birk et al., 1995, 1997; Graham et al., 2000; McBride et al., 1985)). In order to validate the ability of SEM to distinctly image collagen fibril ends, we examined fetal ligaments and tendons since they would be more likely to possess fibril ends for comparison with mature tissues. In addition to fetal rat

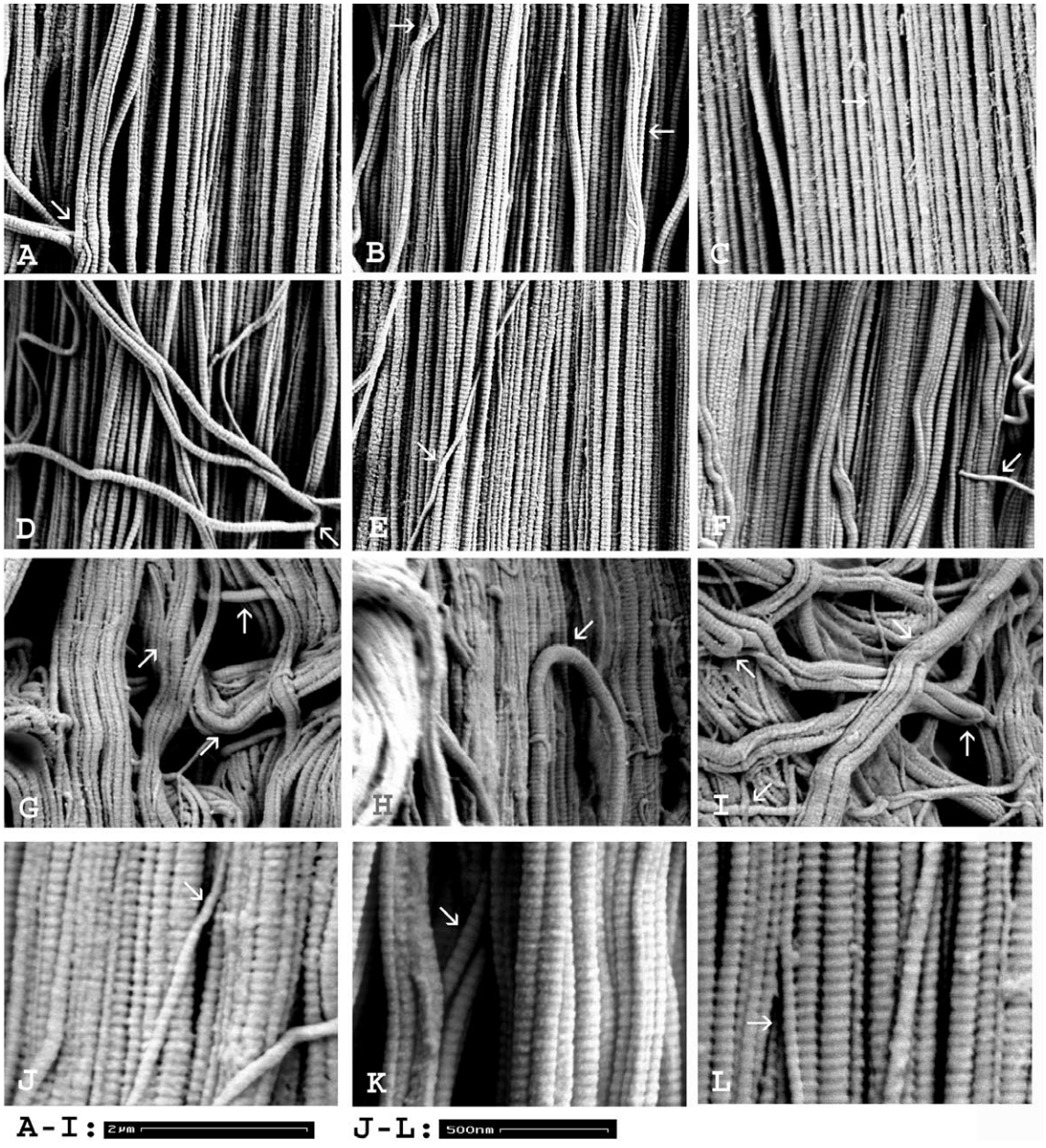

Fig. 3. Collagen fibril organization and morphology in mature rat tendon and ligament. In mature tendon and ligament the collagen fibrils are primarily aligned along the long axis of the ligament, however, substantial interweaving (both with the plane of the image and below the plane of the image), and more randomly organized regions are apparent (A–L). Examples of fibril overlap and interweaving are indicated in: A (arrow), B (arrows), C (arrow, fibril going below the image surface, also see Fig. 6), E (arrow, small diameter fibril traversing larger diameter fibrils), J (arrow), K (arrow), and L (arrow, fibril going below the image plane, also see Fig. 6). In addition, fibril interweaving is clearly visible in D and F–I. Other observed microstructural features are bifurcating/fusing fibrils (left arrow in G, top right arrow in I), fibrils 'turning back' on themselves or looping back (arrow in D, bottom arrow in G, arrow in H, upper left arrow in I, rightmost arrow in I,), fibrils running transverse to the long axis of the tissue (top arrow in G, bottom left arrow I), interweaving fibril which appear 'connected' (bottom left arrow in I), material that is not fibrillar collagen (arrow in F, no banding pattern, appears to wrap around small fibril bundle), and small diameter fibrils traversing larger diameter fibrils (arrow in E). These microstructural features indicate a non-uniform microstructure at this level of organization that will result in a non-uniform load distribution in adjacent fibrils and large non-uniform deformation of fibroblasts between collagen fibrils.

tissues, tissues from fetal bovine and feline animals were chosen because their size during early and late development, respectively, are similar in size to mature rat and therefore more easily facilitate specimen extraction and preparation for scanning electron microscopy, thus reducing artifact. Collagen fibril ends were clearly imaged in tissues from all three species (Figs. 1 and 2). The organization of collagen fibrils within the ground matrix and cellular material during fetal development displays a trend toward fibril alignment in the axial direction of the tendon (Fig. 1), with the characteristic crimp morphology of tendon and ligament present during late development indicating a more organized collagenous matrix. Examination of the collagen fibrils at higher magnification (Fig. 1B–F) clearly indicates the presence of intact tapered fibril ends in all fetal tissues examined. Microscopy reveals collagen fibrils in the extracellular matrix to possess substantial interweaving, and bifurcating/fusing fibrils were clearly visible (Figs. 1 and 2).

Mature ligament and tendon possess long regions in which the collagen fibrils are primarily arranged along the longitudinal axis of the ligament (Fig. 3A–F). However, both ligaments and tendons contain regions that show substantial fibril interweaving, fibrils turning back on themselves, small diameter fibrils traversing and interweaving with larger diameter fibrils, fibril bifurcations/fusions, and portions of fibrils which are not aligned with the long axis of the ligament, but instead travel in the transverse direction (Fig. 3). Hence, although there are regions in which the fibrils are organized in a reasonably homogeneous parallel manner, there are also regions that are non-uniform (i.e. relatively disorganized). These non-axial aspects of fibril morphology, in combination with other phenomena such as fluid transport and intra-fibrillar viscoelasticity, are likely to influence tissue viscoelasticity through kinematic translation of fibrils within fibers, unfolding of curved (interwoven) fibrils, and fibril–fibril slippage. This microstructure may explain why previous reports show fibril elongation to be less than tissue elongation (Fratzl et al., 1997; Puxkandl et al., 2002) and why viscoelastic models have utilized short instead of long fibrils to compensate for the limiting assumption of perfectly aligned, parallel collagen fibrils (Puxkandl et al., 2002). By including kinematic movement of fibrils that are not perfectly aligned through a viscous medium, viscoelastic behavior becomes feasible in long fibril models.

To address the continuity of fibrils within mature ligament and tendon, consecutive sections of tissue were analyzed in addition to individual micrographs. Most fibrils traveled through the micrograph(s), however, some fibrils left the lateral bounds of the image, some were overlapped by other fibrils, and some “dove” below the image plane. To address this behavior, each micrograph was visualized on the microscope at varying levels of brightness and contrast to distinguish between fibrils diving below the plane of the image and any potential fibril ends. Potential ends were carefully examined at multiple magnifications and angles. When examining the overlaid consecutive micrograph it is important to understand that fibrils going below the plane of view can appear to end, but examination at increased brightness clearly shows they continue (Fig. 4). Analysis of groups of collagen fibrils within individual micrographs from mature tendon or ligament did not reveal any fibril ends. Examination of consecutive overlaid micrographs from mature MCLs did not reveal any collagen fibril ends (Fig. 5A,B). In addition, evaluation of consecutive overlaid micrographs from mature tendon did not reveal any collagen fibril ends (Fig. 6A–H). Combined, 7275 fibrils were examined at high magnification (10,000–50,000×) over a total combined tissue length of approximately 2.1 mm without revealing an end in mature tissue. These data show that unlike fetal tissues, virtually all of the collagen fibrils in mature ligament and tendon are very long. These data strongly suggest that collagen fibrils in mature tissues span the length of the tissue.

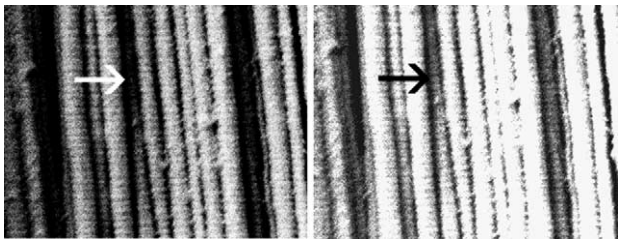

Fig. 4. Example of collagen fibrils that travel out of the plane of the image. During acquisition and analysis of the micrographs, tissues/images were viewed at varying levels of brightness and contrast, to help distinguish collagen fibril ends from fibrils that left the surface plane of the image. The micrograph on the left is representative of the brightness and contrast levels of the micrographs presented in all other figures, while the micrograph on the right is representative of increased brightness. The micrograph on the left shows a fibril which appears to taper and end, but the right image with increased brightness reveals that the fibril is simply leaving the surface plane of the image and diving deeper into the tissue.

3. Discussion

3.1. The structure–function relationship: implications for force transmission

The goal of this study was to elucidate the morphology and organization of collagen fibrils from mature tissues, with a specific emphasis on collagen fibril continuity, to better understand the mechanism of force transmission. Evaluation of collagen fibrils in fetal tendons and ligaments clearly demonstrates the ability of SEM to image undamaged collagen fibril ends as well as numerous fibril bifurcations/fusions (that have been previously reported to be natural structures not resulting from anastomoses during fixation (Provenzano et al., 2001)). When examining tissues from more developed animals (fetal rat and feline tissues and mature rat tissues versus fetal bovine) fibril bifurcations/fusions are present but less evident. Previously, we reported that fibril bifurcations/fusions are present in normal tissue, but are substantially more numerous in scar tissue and at the scar-to-residual tissue junction (Provenzano et al., 2001). These bifurcations/fusions are presumably to connect fibrils in order to transfer force from scar to residual tissue

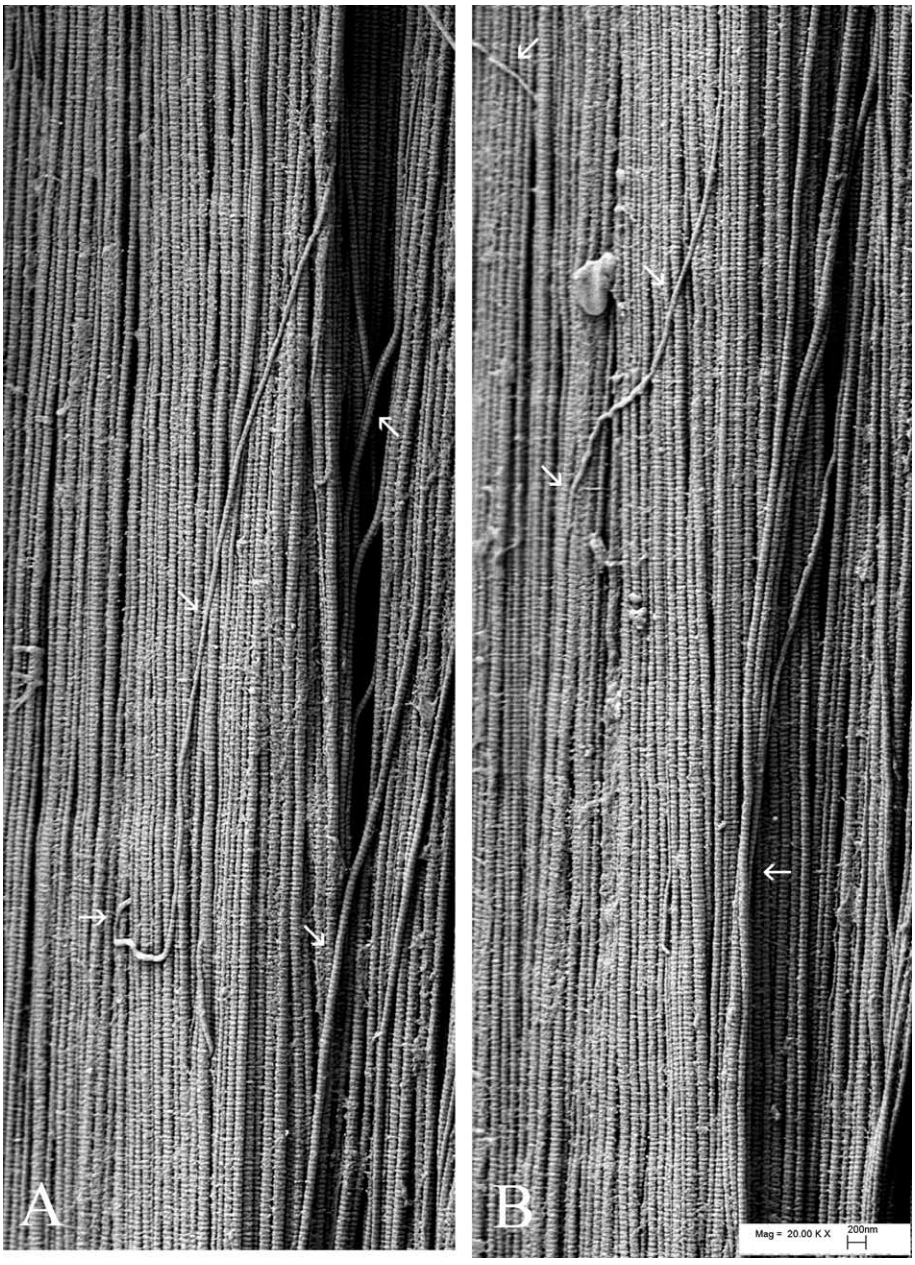

Fig. 5. Collagen fibril continuity in mature ligament. Overlaid consecutive micrographs from mature rat ligament tissue do not reveal any collagen fibril ends (A, B). As described in Figs. 3 and 4, fibrils do overlap and interweave (A: arrows; B: bottom three arrows, top arrow indicates non-fibrillar collagen material) and fibrils do dive below the image plane. In addition, small diameter fibrils traverse larger diameter fibrils and then loop back (A, bottom left arrow) or dive below the surface (B, middle left arrow).

(Provenzano et al., 2001), and may indicate wound healing is an imperfect reversion to fetal development. However, the role of bifurcating/fusing fibrils in normal tissue during early fetal development and their decrease as development progresses is less understood. It is unclear whether these fibrils remain bifurcated/fused as development proceeds, forming an interconnected fibril network that is difficult to observe in mature tissues due to increased fibril density, or if these connected fibrils disconnect later in development to form primarily individual fibrils. Due to the interweaving nature of fibrils in the extracellular matrix of ligament and tendon and the extraordinary strength of these tissues it seems reasonable that interconnected fibrils could aid in force transmission and help provide a basis for structural integrity. Further work is necessary to understand the role and fate of bifurcated/fused fibrils during development and into maturity.

Results of this study using extensive SEM analyses did not identify any fibril ends in ligaments or tendons from mature animals. Neither examination of individual micrographs nor consecutive micrographs identified a single collagen fibril end, precluding our ability to make statistical estimates of length. Surely a small number of ends must be present since the tissue remodels and newly formed fibrils would not immediately appear in a long form. However, we were not able to identify any such fibrils in this study; ends were only

observed in tissues from fetal animals. Hence, in mature tendons and ligaments we conclude that collagen fibrils are very long, likely spanning the length of the tissue with a mean fibril length equal to the tissue length or greater since fibril do not lie in straight lines origin to insertion. This conclusion, supports the concept that force in these tissues is transferred directly through long fibrils. In regard to the theory that force transmission in ligament and tendon occurs via short collagen fibrils connected by load transferring proteoglycans, we feel that this theory is not supported for the following reasons:

1) Results of this study and studies utilizing the transmission electron microscope (Craig et al., 1989; Parry and Craig, 1984; Trotter and Wofsy, 1989) reveal extremely few ends, not supporting the concept of a short fibril. We examined

Fig. 6. Collagen fibril continuity in mature tendon. Overlaid consecutive micrographs from mature rat tendon do not reveal any collagen fibril ends (A–I). Interweaving fibrils are present. A: arrows indicate fibril overlap and/or interweaving, B: arrow indicates a fibril that continues but is difficult to visualize due to changes in brightness/contrast between overlaid images, C: arrows indicate examples of fibrils diving below the image surface, D: arrow further indicates fibril intertwining, E: arrow shows a fibril above the primary image plane which moved due to charging, resulting in that fibril not matching up in adjacent overlaid micrographs, F: arrow indicates a fibril that is looped back and diving below the image plane, G: arrow indicates an adjacent fibril bundle that is overlapping the fibril bundle of interest, H: arrow indicates fibrils that go below the plane of the image as seen in Fig. 4.

Fig. 6 (*continued*).

7275 fibrils without seeing an end. Craig, Parry, and co-workers (Craig et al., 1989; Parry and Craig, 1984) examined 2368 fibrils and did not see an end. Trotter and Wofsy examined 5639 fibrils and found only two ends. However, the fibril ends reported were from small diameter fibrils casting doubt on the maturity of the fibril or whether it is in fact type I collagen.

2) In order for proteoglycans to transfer force between adjacent fibrils, the proteoglycans would need to be stronger than the collagen fibrils (considering fibril rupture can be seen with EM in failed ligaments (Provenzano et al., 2005)) or present in very large concentrations. With respect to proteoglycan shear strength and bond strength, the shear modulus is estimated to be only on the order of 10^{-5} MPa (Hooley and Cohen, 1979; Mow et al., 1984) and the proteoglycan–collagen bond is a very weak, non-covalent bond (Scott, 1988; Scott and Orford, 1981), while the elastic modulus of the collagen fibril is on the order of 430 MPa to >2 GPa (when area corrected) based on molecular measures (Sasaki and Odajima, 1996a,b). This does not intuitively indicate that proteoglycans have enough strength to transfer force between fibrils and break them. However, one could argue that if they were present in very large number that these deficiencies might be overcome. Yet, only a few proteoglycans are present per D-period and if a very large number of proteoglycans were orthogonally bonding adjacent fibrils, the transverse and shear properties of the ligament would be substantial and adjacent fibrils would not easily slide past one another. The transverse and shear strengths are not substantial and since fibrils, and

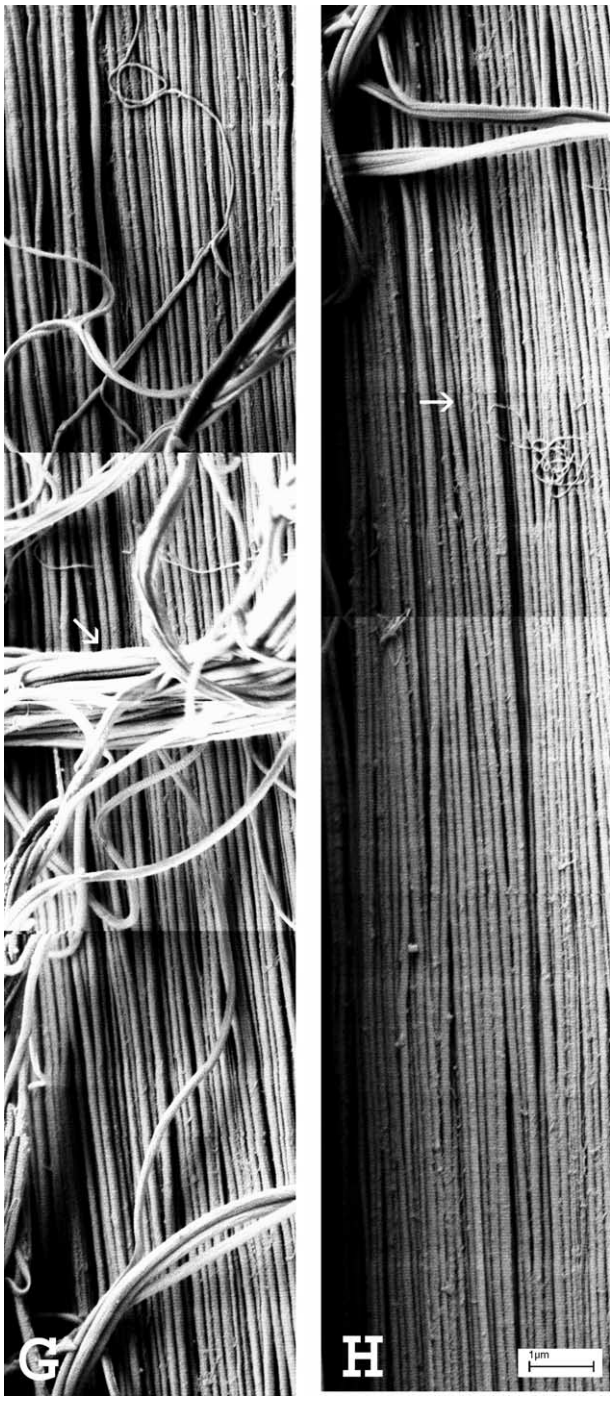

Fig. 6 (*continued*).

groups of fibrils, transition and connect one fiber to another (Danylchuk et al., 1978; Provenzano et al., 2001), failure between fibers can be excluded. The transverse properties of ligaments and tendons are approximately two orders of magnitude lower than the fibril aligned longitudinal properties (Lynch et al., 2003; Stabile et al., 2004) and shear properties are more than three orders of magnitude lower than the fiber aligned properties (Weiss et al., 2002). Moreover, fibrils are known to slide past one another producing local strains different from gross tissue strain (Arnoczky et al., 1994, 2002; Hurschler, 1998; Screen et al., 2003); a behavior inconsistent with proteoglycans coupling fibrils for force transmission.

3) Application of a decorin-inhibiting agent does not reduce the strength of ligament (Caprise et al., 2001).
4) Analysis of the mechanical properties of tendons from decorin deficient mice does not reveal any detrimental effect on the material properties (e.g. maximum load, modulus, etc.) when compared to age matched controls (Forslund et al., 2002; Gimbel et al., 2002; Lin et al., 2002; Robinson et al., 2004), but does reveal rate-dependent properties (Robinson et al., 2004), possibly indicating

changes in hydration, water localization, or the ability of fibrils to slide past one another. In addition, tendons from mice deficient in either lumican or fibromodulin do not show any reduction in elastic modulus (Jepsen et al., 2002). It is not until substantial fibril abnormalities are present in the tendon jointly deficient of lumican and fibromodulin that any change in modulus is detected (Jepsen et al., 2002). Furthermore, the addition of chondoitinase ABC, which removed 90% of the GAGs, did not reduce the maximum force in tendons (Screen et al., 2005).

5) Adding decorin to self-assembled collagen fibers does not significantly increase the mechanical properties of the fibers when compared to controls prepared in the same manner (Pins et al., 1997). Ultimate stress values only slightly increased from 3.500 ± 2.255 to 4.643 ± 2.340 MPa, indicating a mildly positive trend results from adding decorin, but not a significant increase in tensile stress as would be expected if decorin transferred force between fibrils.

Hence, we feel that experimental evidence does not support the concept of force transmission through the extracellular matrix of ligaments and tendon via proteoglycan linkages between fibrils. Alternatively, we feel that strong evidence supports the theory that force transmission within the tissues occurs through long spanning collagen fibrils themselves, long intertwined fibrils, and bifurcating/fusing fibrils. It is abundantly clear that proteoglycans play an important role in collagen fibrillogenesis and matrix hydration, but given the current literature, their role in direct force transmission seems unlikely. It is more likely that proteoglycans aid in tendon and ligament structural integrity by helping to guide fibril maturation, maintaining hydration, and facilitating slippage between adjacent and intertwined fibrils during loading.

3.2. The structure–function relationship: implications for the cell–matrix interaction

Examination of collagen fibril organization in mature ligaments and tendons reveals regions in which undulating and curving fibrils are largely aligned parallel to the long axis of the tissue, but contain fibrils that interweave other fibrils, and regions which are not well aligned. We have previously reported, that cellular necrosis increases as a function of tissue strain in ligament (Provenzano et al., 2002b). This is likely do to high nonlinear cellular deformations resulting form non-uniform matrix deformation. Fibroblasts in ligaments and tendon are primarily found in columns between fibrils, and fibroblasts exhibit invaginations containing single or groups of collagen fibrils (Provenzano et al., 2002b; Squier and Bausch, 1984), which may serve to transmit force/deformation between the fibroblast and the collagen fibrils. Therefore, the organization of the collagen fibril directly affects cellular mechanics. The non-uniform organization of collagen fibrils and thus, the non-uniformly loaded fibroblast environment, help to explain the observation that microstructural strain can be either larger or smaller than macroscopic tissue strains (Hurschler, 1998). Furthermore, since this fibril organization lends itself to non-uniform reorganization and straightening during loading, it presents a possible explanation for observed fibril strains that are much lower than macroscopic tissue strain during tensile loading (Fratzl et al., 1997). Consequently, this fibril non-uniformity causes nonlinear cellular strain and distortion (Arnoczky et al., 2002; Hurschler, 1998) and can cause cellular necrosis at relatively low levels of tissue strain (Provenzano et al., 2002b). Hence, the fibril organization data presented herein indicate that fibrils will not load uniformly, but will be loaded to differing degrees and slip past one another. This extracellular matrix behavior would then result in large local fibroblast strains and nonuniform cellular deformations that have important implications for cellular damage and mechanotransduction in fibroblasts.

4. Conclusion

In conclusion, examination of collagen fibril organization in tendons and ligaments during fetal development reveals interwoven, commonly bifurcating/fusing, fibrils that have not formed fibril bundles. After further development, tendons and ligaments still reveal fibril interweaving and display the characteristic crimp pattern exhibited by collagen fibers, although with a substantially decreased period. In mature animals, fibrils are predominately parallel to axial direction of the tissue but still contain some disorganized regions and are also seen to interweave and bifurcate/fuse. The fibril ends that are clearly visible in fetal tissues are no longer present. Further work is required in order to better image collagen microstructure, define temporal changes such as fibrillogenesis, understand the developmental mechanisms resulting in long fibrils, determine the role bifurcating/fusing fibrils, and elucidate mechanisms by which the extracellular matrix functions in fetal, skeletally immature, and mature tissues.

5. Experimental procedures

This study was approved by the institutional animal use and care committee and meets N.I.H. guidelines for animal welfare. Ligaments and tendons were obtained from fetal day 18 fetal rats (gestation >21 days), fetal day 40 fetal bovine (gestation >280 days), fetal day 50 fetal feline (gestation >60 days), and mature rat (mass ~250 grams). From fetal animals, the medial collateral ligaments and patellar tendons were harvested under a dissecting microscope to provide tissues with fibril ends for comparison with mature tissues, thus validating our ability to detect fibril ends with scanning electron microscopy. Medial collateral ligaments (MCLs) and patellar tendon tissues were harvested from mature rats. Fetal bovine and feline animals were chosen because their size during early and late development, respectively, are similar in size to rat and therefore more easily facilitate specimen extraction and preparation for scanning electron microscopy, thus reducing artifact. In conjunction with fetal rat tissues, this approach allows us to view collagen matrices during development and identify collagen fibril ends (from smaller diameter fibrils in fact),

validating our ability to observe fibril ends in tissues of varying sizes with scanning electron microscopy. All tissues were prepared as previously described (Provenzano et al., 2001). Briefly, animals were placed into a custom designed mold contoured to their body and natural joint angle, after which skin and muscle tissues were transected to expose the ligament or tendon. Intact tissues, in intact anatomically positioned joints, were fixed in situ with 2.5% glutaraldehyde in 0.1 sodium cacodylate buffer, pH 7.4 (GSCB, Electron Microscopy Sciences, Fort Washington, PA) in the intact joint, then harvested and dehydrated through a series of ethanol / H_2O solutions (30%, 50%, 75%, 90% and 100% ethanol). Following dehydration, the tissues were immersed in liquid nitrogen and subsequently placed on a pre-cooled microscope slide. Under a dissecting microscope, tissue fracture, in the sagittal plane, was initiated using a micro-surgical scalpel blade. Samples were then critical point dried (Samdri model 780A, Tousimis Research Corp., Rockville, MA), mounted on 10 mm SEM mounting blocks (JEOL 840, SPI Supplies, Structure Probe, West Chester, PA), gold-palladium sputter coated to ~175 angstroms, and stored in a vacuum container. The tissues were imaged with a scanning electron microscope (LEO 982 or 1530, Leo Electron Microscopy Inc., One Zeiss Drive, Thornwood, NY 10594). Collagen fiber organization was viewed at magnifications of 100–500×. Collagen fibril organization and morphology were examined at 10,000–50,000×. Images were taken at multiple random locations within the tissue. No specific area was imaged, except, when possible, images were taken only slightly below the fracture surface to reduce artifact from preparation and fracture. In fetal animals the organization of the ECM at the fiber level as well as the fibril level is reported. In mature rat tissue, the organization of the ECM at the fibril level is examined as well as the continuity of the fibril. In order to examine the length of the collagen fibrils in mature ligament and tendon, over 400 images from twenty animals were stored and examined between 10,000 and 50,000× (but primarily 10–20 k×) to look for collagen fibril ends. At 10,000, 15,000, 20,000, and 50,000× the micrographs allow groups of fibrils to be examined over lengths of approximately 8, 4, 6, and 1.6 micrometers, respectively. However, the collagen fibrils often have waviness in or out of the plane of the image or do not travel vertically within the micrograph, making exact correlations between the micrograph image window and the length of the fibrils impossible. To assist in the analysis of fibril continuity, 10 to 67 consecutive images were taken of groups of fibrils from eight animals. Micrographs were then overlaid to examine the collagen fibril over a range of lengths. If a potential end was encountered the fibril was examined at multiple magnifications, brightness and contrast settings, and angles to determine if the fibril came to an end or left the plane of imaging or was overlapped by another fibril.

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References

Arnoczky, S.P., Hoonjan, A., Whallon, B., Cloutier, B., 1994. Cell deformation in tendons under tensile load: a morphological analysis using confocal laser microscopy. Trans. Orthop. Res. Soc. 40, 495.

Arnoczky, S.P., Lavagnino, M., Whallon, J.H., Hoonjan, A., 2002. In situ cell nucleus deformation in tendons under tensile load; a morphological analysis using confocal laser microscopy. J. Orthop. Res. 20, 29–35.

Beredjiklian, P.K., Favata, M., Cartmell, J.S., Flanagan, C.L., Crombleholme, T.M., Soslowsky, L.J., 2003. Regenerative versus reparative healing in tendon: a study of biomechanical and histological properties in fetal sheep. Ann. Biomed. Eng. 31, 1143–1152.

Birk, D.E., Trelstad, R.L., 1984. Extracellular compartments in matrix morphogenesis: collagen fibril, bundle, and lamellar formation by corneal fibroblasts. J. Cell Biol. 99, 2024–2033.

Birk, D.E., Trelstad, R.L., 1986. Extracellular compartments in tendon morphogenesis: collagen fibril, bundle, and macroaggregate formation. J. Cell Biol. 103, 231–240.

Birk, D.E., Zycband, E.I., Winkelmann, D.A., Trelstad, R.L., 1989. Collagen fibrillogenesis in situ: fibril segments are intermediates in matrix assembly. Proc. Natl. Acad. Sci. U. S. A. 86, 4549–4553.

Birk, D.E., Nurminskaya, M.V., Zycband, E.I., 1995. Collagen fibrillogenesis in situ: fibril segments undergo post-depositional modifications resulting in linear and lateral growth during matrix development. Dev. Dyn. 202, 229–243.

Birk, D.E., Hahn, R.A., Linsenmayer, C.Y., Zycband, E.I., 1996. Characterization of collagen fibril segments from chicken embryo cornea, dermis and tendon. Matrix Biol. 15, 111–118.

Birk, D.E., Zycband, E.I., Woodruff, S., Winkelmann, D.A., Trelstad, R.L., 1997. Collagen fibrillogenesis in situ: fibril segments become long fibrils as the developing tendon matures. Dev. Dyn. 208, 291–298.

Canty, E.G., Lu, Y., Meadows, R.S., Shaw, M.K., Holmes, D.F., Kadler, K.E., 2004. Coalignment of plasma membrane channels and protrusions (fibripositors) specifies the parallelism of tendon. J. Cell Biol. 165, 553–563.

Caprise, P.A., Lester, G.E., Weinhold, P., Hill, J., Dahners, L.E., 2001. The effect of NKISK on tendon in an in vivo model. J. Orthop. Res. 19, 858–861.

Chakravarti, S., Magnuson, T., Lass, J.H., Jepsen, K.J., LaMantia, C., Carroll, H., 1998. Lumican regulates collagen fibril assembly: skin fragility and corneal opacity in the absence of lumican. J. Cell Biol. 141, 1277–1286.

Craig, A.S., Birtles, M.J., Conway, J.F., Parry, D.A., 1989. An estimate of the mean length of collagen fibrils in rat tail-tendon as a function of age. Connect. Tissue Res. 19, 51–62.

Cribb, A.M., Scott, J.E., 1995. Tendon response to tensile stress: an ultrastructural investigation of collagen: proteoglycan interactions in stressed tendon. J. Anat. 187, 423–428.

Dahners, L.E., Lester, G.E., Caprise, P., 2000. The pentapeptide NKISK affects collagen fibril interactions in a vertebrate tissue. J. Orthop. Res. 18, 532–536.

Danielson, K.G., Baribault, H., Holmes, D.F., Graham, H., Kadler, K.E., Iozzo, R.V., 1997. Targeted disruption of decorin leads to abnormal collagen fibril morphology and skin fragility. J. Cell Biol. 136, 729–743.

Danylchuk, K.D., Finlay, J.B., Krcek, J.P., 1978. Microstructural organization of human and bovine cruciate ligaments. Clin. Orthop. Relat. Res. 131, 294–298.

Derwin, K.A., Soslowsky, L.J., 1999. A quantitative investigation of structure–function relationships in a tendon fascicle model. J. Biomech. Eng. 121, 598–604.

Derwin, K.A., Soslowsky, L.J., Kimura, J.H., Plaas, A.H., 2001. Proteoglycans and glycosaminoglycan fine structure in the mouse tail tendon fascicle. J. Orthop. Res. 19, 269–277.

Ezura, Y., Chakravarti, S., Oldberg, A., Chervoneva, I., Birk, D.E., 2000. Differential expression of lumican and fibromodulin regulate collagen fibrillogenesis in developing mouse tendons. J. Cell Biol. 151, 779–788.

Forslund, C., Aspenberg, P., Iozzo, R., Oldber, A., 2002. Different functions of fibromodulin decorin for tendon strength and maturation. Achilles tendon mechanics in knock-out mice. Trans. 48th Ann. Orthop. Res. Soc., Paper, vol. 0606.

Frank, C., McDonald, D., Bray, D., Bray, R., Rangayyan, R., Chimich, D., Shrive, N., 1992. Collagen fibril diameters in the healing adult rabbit medial collateral ligament. Connect. Tissue Res. 27, 251–263.

Fratzl, P., Misof, K., Zizak, I., Rapp, G., Amenitsch, H., Bernstorff, S., 1997. Fibrillar structure and mechanical properties of collagen. J. Struct. Biol. 122, 119–122.

Gimbel, J.A., Robinson, P.S., Abboud, J.A., Elliott, D.M., Iozzo, R.V., Soslowsky, L.J., 2002. Determining the source of elasticity and viscoelasticity in transgenic mouse tendon fascicles. Trans. 48th Annual Orthop. Res. Soc., Paper, vol. 0603..

Graham, H.K., Holmes, D.F., Watson, R.B., Kadler, K.E., 2000. Identification of collagen fibril fusion during vertebrate tendon morphogenesis. The process relies on unipolar fibrils and is regulated by collagen–proteoglycan interaction. J. Mol. Biol. 295, 891–902.

Holmes, D.F., Chapman, J.A., Prockop, D.J., Kadler, K.E., 1992. Growing tips of type I collagen fibrils formed in vitro are near-paraboloidal in shape, implying a reciprocal relationship between accretion and diameter. Proc. Natl. Acad. Sci. U. S. A. 89, 9855–9859.

Holmes, D.F., Graham, H.K., Kadler, K.E., 1998. Collagen fibrils forming in developing tendon show an early and abrupt limitation in diameter at the growing tips. J. Mol. Biol. 283, 1049–1058.

Hooley, C.J., Cohen, R.E., 1979. A model for creep behavior of tendon. J. Biol. Macromol. 1, 123–132.

Hurschler, C., 1998. Collagen matrix in normal and healing ligaments: microstructural behavior, biological adaptation and a structural mechanical model. In Ph. D. Dissertation: Engineering Mechanics. Univ. of Wisconsin-Madison.

Jepsen, K.J., Wu, F., Peragallo, J.H., Paul, J., Roberts, L., Ezura, Y., Oldberg, A., Birk, D.E., Chakravarti, S., 2002. A syndrome of joint laxity and impaired tendon integrity in lumican- and fibromodulin-deficient mice. J. Biol. Chem. 277, 35532–35540.

Keene, D.R., San Antonio, J.D., Mayne, R., McQuillan, D.J., Sarris, G., Santoro, S.A., Iozzo, R.V., 2000. Decorin binds near the C terminus of type I collagen. J. Biol. Chem. 275, 21801–21804.

Lin, T.W., White, S.M., Robinson, P.S., Derwin, K.A., Plaas, A.H., Iozzo, R.V., Soslowsky, L.J., 2002. Relating extracellular matrix composition with function — a study using transgenic mouse tail tendon fascicles. Trans. 48th Ann. Orthop. Res. Soc., Paper, vol. 0045.

Lynch, H.A., Johannessen, W., Wu, J.P., Jawa, A., Elliott, D.M., 2003. Effect of fiber orientation and strain rate on the nonlinear uniaxial tensile material properties of tendon. J. Biomech. Eng. 125, 726–731.

McBride, D.J., Hahn, R.A., Silver, F.H., 1985. Morphological characterization of tendon development during chick embryogenesis: measurment of birefringence retardation. Int. J. Biol. Macromol. 7, 71–76.

McBride, D.J., Trelstad, R.L., Silver, F.H., 1988. Structural and mechanical assessment of developing chick tendon. Int. J. Biol. Macromol. 10, 194–200.

Mosler, E., Folkhard, W., Knorzer, E., Nemetschek-Gansler, H., Nemetschek, T., Koch, M.H., 1985. Stress-induced molecular rearrangement in tendon collagen. J. Mol. Biol. 182, 589–596.

Mow, V.C., Mak, A.F., Lai, W.M., Rosenberg, L.C., Tang, L.H., 1984. Viscoelastic properties of proteoglycan subunits and aggregates in varying solution concentrations. J. Biomech. 17, 325–338.

Nemetschek, T., Jelinek, K., Knorzer, E., Mosler, E., Nemetschek-Gansler, H., Riedl, H., Schilling, V., 1983. Transformation of the structure of collagen. A time-resolved analysis of mechanochemical processes using synchrotron radiation. J. Mol. Biol. 167, 461–479.

Nimni, M.E., Harkness, R.D., 1988. Molecular structure and functions of collagen. In: Nimni, M.E. (Ed.), Collagen, vol. 1. CRC Press, Boaca Raton, FL, pp. 1–77.

Parry, D.A., Craig, A.S., 1984. Growth and development of collagen fibrils in connective tissue. In: Ruggeri, A., Motta, A. (Eds.), Ultrastructure of the Connective Tissue Matrix. The Hague, Martinus Nijhoff, pp. 34–62.

Pins, G.D., Christiansen, D.L., Patel, R., Silver, F.H., 1997. Self-assembly of collagen fibers. Influence of fibrillar alignment and decorin on mechanical properties. Biophys. J. 73, 2164–2172.

Provenzano, P.P., Hurschler, C., Vanderby, R.J., 2001. Microstructural morphology in the transition region between scar and intact residual segments of a healing rat medial collateral ligament. Connect. Tissue Res. 42, 123–133.

Provenzano, P.P., Hayashi, K., Kunz, D.N., Markel, M.D., Vanderby Jr., R., 2002a. Healing of subfailure ligament injury: comparison between immature and mature ligaments in a rat model. J. Orthop. Res. 20, 975–983.

Provenzano, P.P., Heisey, D., Hayashi, K., Lakes, R., Vanderby Jr., R., 2002b. Subfailure damage in ligament: a structural and cellular evaluation. J. Appl. Physiol. 92, 362–371.

Provenzano, P.P., Alejandro-Osorio, A.L., Valhmu, W.B., Vanderby Jr., R., 2005. Intrinsic fibroblast mediated remodeling of damaged collagenous matrices in vivo. Matrix Biol. 23, 543–555.

Puxkandl, R., Zizak, I., Paris, O., Keckes, J., Tesch, W., Bernstorff, S., Purslow, P., Fratzl, P., 2002. Viscoelastic properties of collagen: synchrotron radiation investigations and structural model. Philos. Trans. R. Soc. Lond., B Biol. Sci. 357, 191–197.

Raspanti, M., Congiu, T., Guizzardi, S., 2002. Structural aspects of the extracellular matrix of the tendon: an atomic force and scanning electron microscopy study. Arch. Histol. Cytol. 65, 37–43.

Redaelli, A., Vesentini, S., Soncini, M., Vena, P., Mantero, S., Montevecchi, F.M., 2003. Possible role of decorin glycosaminoglycans in fibril force transmission in relative mature tendons: a computational study from molecular to microstructural level. J. Biomech. 36, 1555–1569.

Robinson, P.S., Lin, T.W., Reynolds, P.R., Derwin, K.A., Iozzo, R.V., Soslowsky, L.J., 2004. Strain-rate sensitive mechanical properties of tendon fascicles from mice with genetically engineered alterations in collagen and decorin. J. Biomech. Eng. 126, 252–257.

Sasaki, N., Odajima, S., 1996a. Elongation mechanism of collagen fibrils and force–strain relations of tendon at each level of structural hierarchy. J. Biomech. 29, 1131–1136.

Sasaki, N., Odajima, S., 1996b. Stress–strain curve and Young's modulus of a collagen molecule as determined by the X-ray diffraction technique. J. Biomech. 29, 655–658.

Scott, J.E., 1988. Proteoglycan–fibrillar collagen interactions. Biochem. J. 252, 313–323.

Scott, J.E., 1996. Proteodermatan and proteokeratan sulfate (decorin, lumican/fibromodulin) proteins are horseshoe shaped. Implications for their interactions with collagen. Biochemistry (Mosc). 35, 8795–8799.

Scott, J.E., Orford, C.R., 1981. Dermatan sulphate-rich proteoglycan associates with rat tail-tendon collagen at the d band in the gap region. Biochem. J. 197, 213–216.

Scott, J.E., Orford, C.R., Hughes, E.W., 1981. Proteoglycan–collagen arrangements in developing rat tail tendon. An electron microscopical and biochemical investigation. Biochem. J. 195, 573–581.

Screen, H.R.C., Shelton, J.C., Bader, D.L., Lee, D.A., 2003. The effects of non-collagenous matrix components on tendon fascicle micromechanics. Trans. 49th Ann. Ortho. Res. Soc., Paper, vol. 0806.

Screen, H.R., Shelton, J.C., Chhaya, V.H., Kayser, M.V., Bader, D.L., Lee, D.A., 2005. The influence of noncollagenous matrix components on the micromechanical environment of tendon fascicles. Ann. Biomed. Eng. 33, 1090–1099.

Squier, C.A., Bausch, W.H., 1984. Three-dimensional organization of fibroblasts and collagen fibrils in rat tail tendon. Cell Tissue Res. 238, 319–327.

Stabile, K.J., Pfaeffle, J., Weiss, J.A., Fischer, K., Tomaino, M.M., 2004. Bi-directional mechanical properties of the human forearm interosseous ligament. J. Orthop. Res. 22, 607–612.

Svensson, L., Aszodi, A., Reinholt, F.P., Fassler, R., Heinegard, D., Oldberg, A., 1999. Fibromodulin-null mice have abnormal collagen fibrils, tissue

organization, and altered lumican deposition in tendon. J. Biol. Chem. 274, 9636–9647.
Trotter, J.A., Wofsy, C., 1989. The length of collagen fibrils in tendons. Trans. Orthop. Res. Soc. 14, 180.
Vogel, K.G., Trotter, J.A., 1987. The effect of proteoglycans on the morphology of collagen fibrils formed in vitro. Coll. Relat. Res. 7, 105–114.
Weber, I.T., Harrison, R.W., Iozzo, R.V., 1996. Model structure of decorin and implications for collagen fibrillogenesis. J. Biol. Chem. 271, 31767–31770.
Weiss, J.A., Gardiner, J.C., Bonifasi-Lista, C., 2002. Ligament material behavior is nonlinear, viscoelastic and rate-independent under shear loading. J. Biomech. 35, 943–950.
Woo, S.L., Orlando, C.A., Gomez, M.A., Frank, C.B., Akeson, W.H., 1986. Tensile properties of the medial collateral ligament as a function of age. J. Orthop. Res. 4, 133–141.