Molecular structure of tail tendon fibers in TIEG1 knockout mice using synchrotron diffraction technology

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1Biomécanique et Bioingénierie, Université de Technologie de Compiègne, Compiègne; 2Laboratoire de Physique des Solides, Université Paris-Sud, Orsay, France; 3Department of Biochemistry and Molecular Biology, Mayo Clinic, Rochester, Minnesota; and 4Centre de Biophysique Moléculaire, Université d’Orléans, Orléans, France

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Gumez L, Bensamoun SF, Doucet J, Haddad O, Hawse JR, Subramaniam M, Spelsberg TC, Pichon C. Molecular structure of tail tendon fibers in TIEG1 knockout mice using synchrotron diffraction technology. J Appl Physiol 108: 1706–1710, 2010. First published April 8, 2010; doi:10.1152/japplphysiol.00356.2010.—The purpose of this study was to characterize the effect of TIEG1 on the molecular structure of collagen within tail tendon fibers using 3-month-old female C57BL/6 wild-type (WT) and TIEG1 KO mice. Synchrotron X-ray microdiffraction experiments were carried out on single tendon fibers extracted from the WT and TIEG1 KO dorsal tail tendons. The fibers were scanned in the radial direction, and X-ray patterns were obtained. From these patterns, the meridional direction was analyzed through X-ray intensity profile. In addition, collagen content was investigated using hydroxyproline assays, and qualitative real-time PCR experiments were performed on RNA isolated from fibroblasts to examine specific gene expression changes. The results showed different X-ray diffraction patterns between WT and TIEG1 KO tendon fibers, indicating a disorganization of the collagen structure for the TIEG1 KO compared with WT mice. Furthermore, the analyses of the X-ray intensity profiles exhibited a higher (23 Å) period of collagen for the TIEG1 KO compared with WT mice. The results of the hydroxyproline assays revealed a significant decrease in the total amount of collagen present within the TIEG1 KO compared with the WT mice. Moreover, qualitative real-time PCR results showed differences in the expression profiles of specific genes known to play important roles in tendon fiber development. These data further elucidate the role of TIEG1 on tendon structure and could explain the previous defects in the structure-function relationship found for TIEG1 KO tendon fibers.

X-ray; hydroxyproline; collagen organization; qualitative real-time polymerase chain reaction

TRANSFORMING GROWTH FACTOR (TGF)-β inducible early gene-1 (TIEG1) is a member of the Krüppel-like family of transcription factors (KLF10) and encodes a 480-amino acid protein (72 kDa) (36). This protein is expressed in multiple cell types including osteoblasts, fibroblasts, lung epithelial and heart cells, as well as in many tissues such as the pancreas, liver, muscle, breast, and pancreatic carcinomas (6, 9, 16–18, 28, 34, 36–38). TIEG1 is induced by several members of the TGF-β superfamily (18, 36) and has been shown to be a critical mediator of TGF-β activity by inducing the expression of Smad2 and inhibiting the expression of Smad7 (22). TIEG1 has also been implicated in the regulation of important osteoblast marker genes, and osteoblasts isolated from TIEG1 knockout mice display severe defects in their ability to differentiate and mineralize in culture (35).

The role of the tendon is to transmit the force developed by the muscle to the bone (29). This connective tissue has a hierarchical structure and contains fascicles that are full of collagen fibrils (33) and organized longitudinally, transversally, and horizontally (24). The tendon is a complex tissue containing few fibroblasts and an extracellular matrix, which is mainly composed of collagen type I and long chains of amino acids such as proteoglycans and glycoproteins (26, 29).

Many studies have characterized the structure-function relationships of the tendon by performing mechanical tests on altered matrix proteins (proteoglycan) from tail tendon fascicles (13). Different stress relaxation properties (viscoelasticity) were found between knockout mice having defect in decorin and control mice, suggesting the great importance of the proteoglycan in the structure-function relationships in tendon. Furthermore, the glycosaminoglycans were found to be the strongest predictor of the mechanical properties in tendon (31). In previous studies (2), mechanical tests were performed on tendon fibers to characterize the effects of TIEG1 on their mechanical properties. The TIEG1 KO mouse tendons had reduced mechanical strength with a lower fibril density than those of the control group. The continuity of this structure-function relationship is to further analyze the tendon matrix by investigating the molecular structure of collagen.

Electron micrograph acquisitions have characterized the collagen structure of the tendon, composed of an alternating dark and light band, defined by a period (D) of 67 nm (670 Å) (7, 19), which may vary according to the type of tissue (D_{ligament} = 67 nm; D_{tendon} = 65 nm) (5, 8). In addition, X-ray diffraction studies using synchrotron radiation allows us to investigate the microstructure of the collagen matrix within the tendon (5, 20). Many studies have combined in situ mechanical tests and X-ray diffraction simultaneously to correlate the molecular mechanism with the structure of collagen (5, 15, 25). Moreover, numerical models are developed to simulate the behavior of the molecular changes under different strains. These models allow for a better understanding of the interaction between the internal structure of collagen within a tendon and its extracellular matrix (27, 30, 32, 39).

To further understand the role of TIEG1 in the structure-function relationship of tendon fibers, we sought to 1) characterize the effects of loss of TIEG1 expression on the molecular structure of collagen (triple helix, intra and inter fibril structure) using synchrotron X-ray microdiffraction experiments on tendon fibers isolated from TIEG1 KO mice, 2) determine the
isolate viable fibroblast cells from mouse tail tendons have been unsuccessful in contrast to the success reported when the larger rat model was used. We also found that a sufficient amount of high-quality RNA could be isolated directly from mouse flexor tendon tissue.

Synchrotron technique. Microdiffraction experiments were carried out at the European Synchrotron Radiation Facility beamline ID13 (Grenoble, France) using a 13-KeV monochromatic beam 2 μm in diameter. Each single tendon fiber (NWT = 9 and NTIEG1_KO = 8) was introduced into a capillary (diameter = 0.7 mm) and placed vertically in the X-ray beam. Diffraction data arising from the internal supramolecular architecture of the tendon fiber were collected on a two-dimensional detector (MARCCD camera).

X-ray patterns from WT (Fig. 1A) and TIEG1 KO (Fig. 1B) fibers were obtained by scanning the fibers along the radial direction (160 μm). Then the beam was translated along the fiber to repeat the scan two to three times. Figure 1C shows a meridian profile drawn from the center to one extremity of the X-ray pattern to obtain an X-ray profile intensity (Fig. 2). The X-ray intensity along the meridional direction (fiber axis) was extracted for detailed analysis of the molecular packing, allowing for determination of the period (D) of the collagen fiber (Fig. 2).

In addition to the period, the lateral organization of collagen triple helix was characterized with a lateral profile placed on the X-ray pattern (Fig. 1C). In parallel to this study, a numerical model was used to analyze the molecular structure of the collagen triple helix recorded along the lateral direction (12). This model provides a percentage of distortion that is dependant on the distance between two triple helices and its standard deviation.

Hydroxyproline content. Hydroxyproline assays were performed to estimate the collagen content within the dorsal tail tendon. The tendon samples were incubated overnight at a temperature of 110°C in 1.5 ml

hydroxyproline content with a goal of estimating collagen amount within these tendon fibers, and 3) identify specific genes that may be in part responsible for the changes observed in tendon structure using quantitative real-time PCR.

MATERIALS AND METHODS

Animals. TIEG1 KO mice were developed in a C57BL/6 background as described previously by Subramaniam et al. (35). All mice were housed in a temperature-controlled room (22 ± 2°C) with a daily light/dark cycle of 12 h. All animals had free access to water and were fed standard laboratory chow (Laboratory Rodent Diet 5001; PMI Feeds, Richmond, VA). To reduce variability, wild-type and KO littermates were used for all experiments performed in the present report. The Institutional Animal Care and Use Committee approved all animal procedures.

Tendon and tendon fiber preparation. A total of 14 WT and 13 TIEG1 KO 3-mo-old C57BL/6 female mice were utilized for these studies. All mice were killed with CO₂ and stored at -80°C until time of study. Tails were amputated close to the body attachment, and the dorsal tail tendon was located using a stereomicroscope. Five WT and four TIEG1 KO mice were used for the synchrotron technique, and six WT and six TIEG1 KO mice were used for the biochemical analysis. For the synchrotron technique, approximately two to three tendon fibers were harvested per mouse from the dorsal tendon. Hydroxyproline assays were performed on the dorsal part of the tail tendon, which was extracted along a length of 1 cm from WT and TIEG1 KO mice. The small piece of tendon was cleaned of muscle and immediately weighed for normalization purposes.

Cell culture. Approximately 18 flexor tendons were harvested from the paws of each of three WT and three TIEG1 KO female mice, and viable fibroblasts were isolated and cultured. The tendons were digested in collagenase type I (0.5 mg/ml) overnight, and the isolated fibroblasts were cultured in αMEM medium containing 10% fetal bovine serum (Invitrogen, France), 100 U/ml penicillin, 100 mg/ml streptomycin, and 0.25 mg/ml Fungizone (Invitrogen, France). The cells were grown at 37°C in a 5% CO₂ humidified atmosphere. We chose to use RNA isolated from cultured fibroblasts extracted from the flexor tendons of WT and TIEG1 KO mice since our attempts to

Fig. 1. Tendon fiber X-ray pattern for wild-type (WT; A) and TIEG1 knockout (KO) (B) mice. C: profiles drawn along the meridional and lateral directions. D: order of the peak represented on the meridional X-ray intensity profile.

Fig. 2. Meridional X-ray intensity (I) for WT and TIEG1 KO tendon fibers represented in arbitrary units (au) and depending on the scattering (S). The peaks of intensity are labeled with a number corresponding to the order of the peak obtained in the X-ray pattern. The distance between two peaks represent the period D.
Table 1. Weight, normalized hydroxyproline, and collagen content of WT or TIEG1 KO dorsal tail tendon

<table>
<thead>
<tr>
<th>Weight of tendon, mg</th>
<th>Hydroxyproline: µg/mg of tendon</th>
<th>Collagen: µg/mg of tendon</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT</td>
<td>2.62 ± 1.47</td>
<td>0.52 ± 0.10</td>
</tr>
<tr>
<td>Median</td>
<td>2.05</td>
<td>0.49</td>
</tr>
<tr>
<td>TIEG1 KO</td>
<td>3.67 ± 1.65</td>
<td>0.35 ± 0.04</td>
</tr>
<tr>
<td>Median</td>
<td>3.50</td>
<td>0.36</td>
</tr>
</tbody>
</table>

Values are means ± SD and median of the weight, normalized hydroxyproline, and collagen content measured using a 1-cm length of wild-type (WT) or TIEG1 knockout (KO) dorsal tail tendon.

of perchloric acid solution. Then hydrolysis was made, and the solution was dissolved in 8.5 ml of water. A colorimetric determination was made using Chloramine T and Erlich reagent. The color density was quantified at 558 nm by using a spectrophotometer (Varian Cary 50 Scan UV-Visible) to determine the total hydroxyproline amount for each sample. This amount was normalized in function of the tendon weight. According to the literature (10, 11), hydroxyproline represents 13% of collagen allowing us to estimate its content.

Quantitative real-time PCR. Quantitative real-time PCR (qRT-PCR) was performed on fibroblast cell cultures using the TaqMan Gene Expression Cells-to-Ct kit as specified by the manufacturer (Applied Biosystems). Briefly, cultured fibroblasts cells were detached from the flask with trypsin, counted, and resuspended at a concentration of 200 cells/µl in lysis solution containing 1% DNase I. The lysis reaction was stopped using stop solution, and cDNA was synthesized via reverse-transcription PCR using the supplied master mix with the following conditions: a first step of 37°C for 60 min, followed by a second at 95°C for 5 min, and a final holding step of 4°C.

Real-time PCR was subsequently performed using gene-specific primers and a MiniOpticon real-time PCR detection system (Bio-Rad) with the following thermal cycling conditions: an initial step of 2 min at 50°C, an activation step of 95°C for 10 min followed by 40 cycles of 95°C for 15 s, and 60°C for 1 min using a PCR cocktail composed of 2× TaqMan gene expression master mix (Cells-to-Ct kit, Applied Biosystems) and 20× TaqMan gene expression assay (Applied Biosystems). Gene-specific primers were purchased from Applied Biosystems, and the reverse primer sequences used for the target genes were as follows: 1) Collagen Iα2 (Col1α2): GCA ACA TGC CAA TAT TIG CAA TCAGG (Mm00438388_m1); 2) Decorin (Dcn): TCC CTC CAA AAC GTG TGG CTA CTC CTCT (Mm00514535_m1); 3) Fibromodulin (Fmod): ACA GGA TCA ATG AGT TCT CCA TCAG (Mm00491215_m1); 4) TIEG1 (Klf10): AGC AAC TCT CCG AGG AGG AAA TGG GGA (Mm00449812_m1); 5) Laminin beta1–1 (Lamb1–1): CCA AAA CCT GCT AAC ATC GAT TGAA (Mm00801853_m1); 6) Lysyl oxidase (Lox): TGC ACT GCA CAC ACA CAG GGA TTGA (Mm00495386_m1); and 7) Lumican (Lum): CTT GAA AAG TTT GTG AAG AGCT (Mm01248292_m1). The housekeeping gene used was beta-2 microglobulin (B2m): GGC CTG TAT GTC CAG AAA ACCC (Mm00437762_m1). Quantitative values were calculated based on the threshold cycle (Ct) obtained during the real-time PCR reaction. The ratio of each target gene was calculated using a relative quantification method: 2^(-ΔΔCt) (Livak, 2001). Two independent experiments were performed in duplicate.

Statistical analysis. Unpaired t-tests were performed with the software Statgraphics 5.0 (Sigma Plus) to compare the hydroxyproline content between the WT and TIEG1 KO mice and the gene expression differences between WT and TIEG1 KO fibroblasts at a 95% confidence interval.

RESULTS

X-ray diffraction patterns analyses. Figure 1 shows the results of the X-ray patterns obtained for the WT (Fig. 1A) and TIEG1 KO mice (Fig. 1B). Within each tendon fiber group isolated from the same genotype (TIEG1 KO vs. WT), the same X-ray patterns were observed. The comparison between X-ray diffraction patterns from WT and TIEG1 KO exhibited significant differences, reflecting a different fibril structure organization between WT and TIEG1 KO mice.

X-ray intensity along the meridian. Figure 2 illustrates the intensity profiles along the meridian (Fig. 1C) associated with the X-ray patterns of Fig. 1, A and B. The series of sharp peaks correspond to the various orders (from orders 3 to 9) of the characteristic periodicity of collagen (Fig. 1D). For the WT tendon fibers, the highest intensity is observed for the sixth order peak, whereas it corresponds to the fifth order for the TIEG1 KO. In addition, the TIEG1 KO tendon fibers have a higher period (D = 673 Å) compared with the WT (D = 650 Å).

Lateral study of the X-ray pattern. The X-ray diffraction patterns from TIEG1 KO mice (Fig. 1B) showed a characteristic diffuse “butterfly” shape that did not appear for the WT mice. The result of the lateral analysis (Fig. 1C) showed a significant distortion of the triple helix network for the TIEG1 KO mice compared with the WT mice.

Hydroxyproline and collagen content. The results of these experiments are summarized in Table 1. A large range of weight was found for the dorsal piece of tendon extracted for both the WT (from 1 to 4.9 mg) and TIEG1 KO (from 1.8 to 5.6 mg) mice. This variation is due to the intrinsic morphology of the mice even though the size and the weight of the WT and TIEG1 KO mice were equivalent.

The hydroxyproline content was significantly decreased in tendons isolated from TIEG1 KO mice relative to WT controls (~0.13 µg/mg), leading to a significant decrease (P < 0.001) in the amount of collagen in TIEG1 KO tendons (Table 1).

qRT-PCR. The results of qRT-PCR revealed that lumican, laminin beta1-1, and decorin gene expression levels are significantly (P < 0.05) decreased in TIEG1 KO fibroblasts relative to WT controls, whereas collagen I expression is significantly increased (Fig. 3). The ratios of lysyl oxidase and fibromodulin are similar between WT and TIEG1 KO fibroblasts. In addition and as a control, the expression of TIEG1 (Klf10) was not detectable in TIEG1 KO fibroblasts.
DISCUSSION

Previously, it has been shown that TIEG1 plays an important role in the growth, morphology, and mechanical properties of tendons. Indeed, we have previously demonstrated that the lack of TIEG1 expression increases the diameter and decreases the strength of 3-mo-old tendon fibers (2). To further explain this structure-function relationship, various aspects concerning the impact of TIEG1 on the internal structure of tendons were investigated: 1) is the molecular organization of the collagen modified; 2) are there changes in collagen content; 3) are there different types of molecules constituting TIEG1 KO tendon fibers relative to WT controls; and 4) are there gene expression differences that may be responsible for the observed differences in collagen modification?

In the present study, we have characterized the molecular organization of TIEG1 KO tendon fibers using synchrotron analysis. X-ray analysis of WT tendon fibers revealed a similar period (D = 650 Å) as it has been reported in the literature previously (5). However, the TIEG1 KO period (D = 673 Å) is significantly increased by 23 Å, demonstrating that the organization of tendon fiber structure is different between TIEG1 KO and WT mice. These data lend evidence for differences in molecular packing of collagen molecules between WT and TIEG1 KO fibers. Moreover, this result suggests that the lack of TIEG1 expression could result in modifications to the internal organization of tendon fiber structure and may explain the decrease in the mechanical properties of TIEG1 KO tendon fibers (2). In addition, modification of the internal structure is also represented by the butterfly shape exhibited only in the TIEG1 KO tendon fibers. This pattern was further investigated using a numerical model (12) analyzing the molecular organization of the collagen in the lateral direction, within the triple helix of collagen. The higher disorder parameter found for the TIEG1 KO tendons reflects a disorganization of the collagen network and could explain the difference of periodicity found between the WT and TIEG1 KO mice. The similar distance obtained between the triple helix of collagen for WT and TIEG1 KO mice reinforces the conclusion that the structure-function relationship could be explained by a molecular disorganization present within the internal structure of the TIEG1 KO tendon fiber.

The second point of investigation was to determine whether differences in the amount of collagen content could be detected in tendons of TIEG1 KO mice. Our analysis revealed a significant decrease in the amount of hydroxyproline content, reflecting a decrease in total collagen content, in TIEG1 KO tendons. This difference in collagen content could lead to the decreases in tendon strength that we have previously reported (2).

Although biochemical methods can be used to characterize the collagen structure and the matrix composition of tendons as well as the amount of collagen (hydroxyproline), the level of collagen cross-linking (lysyl oxidase), proteoglycan content, and sulphated glycosaminoglycan content (10, 11), we sought to further examine changes in the expression levels of genes known to be involved in tendon structure and strength. These data can also yield insights into defects in tendons at the molecular level. Such genes, including decorin, lumican, and fibromodulin, belong to the small leucine-rich proteoglycan (SLRP) family and have previously been implicated in mediating tendon structure. More specifically, decorin interacts with TGF-β and influences collagen fibrillogenesis, and alterations in its expression levels could lead to disorganization of the tendon structure (4). Lumican regulates collagen fibril organization and the circumferential growth of tendons, which is an essential process in tendon development (14). Fibromodulin is known to participate in the assembly of the extracellular matrix through its interaction with both type I and type II collagen fibrils and has been suggested to regulate TGF-β activity (1, 14). Laminins, including Laminin beta-1, are the major non-collagenous constituents of basement membranes and therefore play important roles in mediating tendon structure (23). Moreover, type-I collagen is the most abundant collagen found in tendons (21). Therefore, alterations in the expression of these tendon-related genes could significantly affect tendon structure, microarchitecture, and function.

Interestingly, we demonstrate that loss of TIEG1 expression results in significant decreases in the expression levels of lumican, laminin beta-1, and decorin, as well as a significant increase in the expression of type-I collagen, in tendon fibroblasts. Although the exact mechanisms are not yet clear, these data suggest that TIEG1 can either directly or indirectly regulate these genes and provide a potential explanation for the tendon phenotype described previously in TIEG1 KO animals (2) as well as the structural changes identified here.

The present study has allowed us to better understand the effects of TIEG1 on the molecular organization of collagen and on the collagen matrix composition in tendons. The results of this study should also allow us to further our knowledge concerning the role of TIEG1 in the structure of the musculoskeletal system. Furthermore, the design of new therapeutic approaches for musculoskeletal disorders related to TIEG1 or other members of the TGF-β superfamily could be developed.

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Molecular Structure of Tail Tendon Fibers in Tieg1 KO Mice


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