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Age-dependent changes in the mechanical properties of tail tendons in TGF- β inducible early gene-1 knockout mice

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Bensamoun, Sabine F., Tetsu Tsubone, Malayannan Subramaniam, John R. Hawse, Emir Boumediene, Thomas C. Spelsberg, Kai-Nan An, and Peter C. Amadio. Age-dependent changes in the mechanical properties of tail tendons in TGF- β inducible early gene-1 knockout mice. *J Appl Physiol* 101: 1419–1424, 2006. First published June 22, 2006; doi:10.1152/jappphysiol.00800.2005.—The purpose of this study is to investigate age-dependent changes in the architecture and mechanical properties of tendon in TGF- β inducible early gene-1 (TIEG) knockout mice. Wild-type and TIEG knockout mice, aged 1, 2, and 15 mo, were used. The mechanical properties of tail tendons isolated from these mice were determined using uniaxial tensile ramp (0.05 mm/s) and relaxation (5 mm/s) tests, with a strain of 10%. Mechanical parameters (Young's modulus from the ramp test; fast and static stresses from the relaxation test) were measured and recorded. The structure of the tail tendon fascicle was characterized by transmission electron microscopy. The results of the mechanical testing revealed no significant difference between the knockout and wild-type groups at 1 or 15 mo of age. However, the fascicles of the knockout mice at 3 mo of age exhibited decreased fast and static stresses compared with those of the wild-type mice. Electron microscopy revealed an increase in fibril size in the knockout mouse tendons relative to wild-type controls at 1 and 3 mo of age. These data indicate an important role for TIEG in tendon microarchitecture and strength in adult mice.

tensile strength; tendon fibrils

TENDONS ARE COMPLEX TISSUES composed of type 1 collagen aggregations, elastin, proteoglycans, glycolipids, water, and cells that connect muscle to skeletal structures. Tendon is the densest form of collagenous supporting tissue, consisting of bundles of collagen fibers that play an important role in the tensile properties of this tissue (8, 17). It has been demonstrated that the mechanical properties of collagen fascicles in the patellar tendons of rabbits change with age (20). It has also been shown that there is a strong correlation between ground substance material and the mechanical properties of collagen fascicles (18). It is well known that TGF- β and other growth factors, including IGF-I, PDGF, and bFGF, have been implicated in collagen production, cell migration, cell proliferation, and tendon healing (11). TGF- β is a cytokine that plays an important role in the development and regulation of differentiation and apoptosis in various cell types, including epithelial cells, osteoblasts, and fibroblasts (3). The presence of TGF- β is also known to enhance the repair of many types of injured tissues. Recent studies have demonstrated that application of TGF- β to the patellar tendon of rabbits significantly increases

the tangent modulus and the tensile strength, suggesting that TGF- β plays an important role in the mechanical properties of tendons (1, 19).

TGF- β is well known to regulate the expression of many genes in various cell types. One particular gene, TGF- β inducible early gene-1 (TIEG), was originally identified in human osteoblasts as transcript that is induced rapidly by TGF- β and BMP2 treatment (15). The TIEG gene encodes a 480 amino acid protein that is classified as a member of the Krüppel-like family of transcription factors (3). TIEG plays an important role in Smad signaling by downregulating the expression of Smad 7, resulting in the overall enhancement of this pathway (7). TIEG has recently been shown to play a role in the regulation of cell proliferation and apoptosis (2, 12, 16). To better understand the biological function of TIEG, we have created a TIEG knockout mouse. Initial characterization of these mice has revealed significant defects in the actions of both osteoblasts and osteoclasts (14).

Growth factors, including TGF- β , play important roles in the production of collagen, cell migration, and cell proliferation in tendons (11) and is involved in tendon healing and strength (1, 9). Based on the fact that TIEG is a primary response gene induced by TGF- β , which plays an important role in this signaling cascade, it was of interest to determine whether TIEG had any effect on the organization and mechanical properties of tendons by using our TIEG knockout mouse model. For this study, we chose to examine 1- (adolescent), 3- (adult), and 15-mo-old (aged) animals to determine whether changes in tendons occurred throughout age or only during a particular stage of life.

MATERIALS AND METHODS

Animals

To better understand the biological role of TIEG, we developed TIEG knockout mice (TIEG^{-/-}) (14). Briefly, TIEG-deficient embryonic stem cells were developed in collaboration with Incyte Genomics (St. Louis, MO) by targeted disruption of 2.3 kb of the TIEG promoter and 5'-flanking sequences including exons 1 and 2, and a portion of exon 3. Mice homozygous for this disruption fail to express TIEG mRNA and protein (14).

Fascicle Preparation

A total of 25 C57 black6/129 female mice, 12 wild-type (TIEG^{+/-}), and 13 knockout (TIEG^{-/-}) mice were euthanized at 1 (M1), 3 (M3), or 15 (M15) mo of age. Four wild-type mice ($n = 4$; average

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weight = 15.7 ± 1.3 g) and four knockout mice ($n = 4$; average weight = 15.6 ± 1.4 g) were analyzed at 1 mo of age. Five wild-type mice ($n = 5$; average weight = 20.4 ± 1.7 g) and five knockout mice ($n = 5$; average weight = 21.1 ± 1.5 g) were used for the 3-mo time point. Four wild-type mice ($n = 4$; average weight = 33.3 ± 5.4 g) and three knockout mice ($n = 3$; average weight = 29.9 ± 3.2 g) were used for the 15-mo time point. These mice were killed at the indicated age points with CO_2 . The tails of each mouse were amputated close to the body attachment and were stored at -80°C until ready for study. Under a surgical microscope, fascicles were extracted from the proximal part of the dorsal tail tendon. Approximately four to five fascicles per mouse were harvested.

Mechanical Measurements

Experimental setup. The fascicle to be tested was mounted horizontally under a binocular microscope in a chamber containing a saline solution maintained at a constant temperature of $25 \pm 1^\circ\text{C}$. The extremities of the fascicles were glued with cellulose polyacetate between two hooks, which were connected to a load cell (1.5 N, GS0-50, Transducer Techniques) and to a linear servomotor (MX 80 Daedal), respectively. The displacement of the stage and the developed forces of the fascicles under stresses were measured and recorded through a custom-made LABVIEW program (National Instruments, Austin, TX).

Preload of the fascicles. The fascicles were not preconditioned by a cyclic loading but were preloaded with a small stretch in a range of 15–30 mN to simulate in situ conditions. Following preload, the length (L_0), which corresponds to 100% of the fascicle length, was measured with a micrometer (magnification $\times 40$) through the binocular microscope. The cross section of the fascicle was assumed to be circular, and the diameter was determined as an average of 10 measurements taken along the fascicle axis in one direction with the same binocular microscope ($\times 40$). No optical device was used to measure these parameters; however, no slippage of the sample around the grip was noticed.

Mechanical tests. Two different mechanical tests were performed.

RAMP STRETCH. The ramp stretch test was performed by stretching the fascicle with a slow velocity (0.05 mm/s) up to 110% L_0 , at which point it was released with the same velocity back to its original length, L_0 . This stretch did not continue until failure, so we could perform a second mechanical test on the same fiber. The force-displacement curve was recorded, and the stress-strain curve was calculated, allowing us to measure Young's modulus (E) (Fig. 1) from the slope of the linear portion of the curve with a custom Matlab program.

RELAXATION TEST. For the relaxation test, the fascicle was stretched to 110% L_0 with a faster velocity of 5 mm/s, and this length was maintained for 60 s before the fascicle was released back to its original length. This stretch-release protocol enabled us to measure the maximal force developed at a fast rate ramp and the static force of the fascicle at the end of the 60-s period. Then, fast rate ramp and static force were divided by the cross-sectional area to obtain a measure of the fast (σ_f) and static (σ_s) stresses, respectively (Fig. 2). The viscoelastic stress relaxation was expressed as a percentage of the ratio between σ_f and σ_s .

Transmission Electron Microscopy

One-centimeter lengths of wild-type and TIEG^{-/-} tail tendons at 1 mo ($n^{+/+} = 4$; $n^{-/-} = 4$), 3 mo ($n^{+/+} = 5$; $n^{-/-} = 5$), and 15 mo ($n^{+/+} = 4$; $n^{-/-} = 3$) of age were fixed in Trump's fixative [1% (vol/vol) glutaraldehyde and 4% (vol/vol) formaldehyde in 0.1 M phosphate buffer, pH 7.2] (10) and rinsed for 30 min in three changes of 0.1 M phosphate buffer, pH 7.2, followed by a 1-h postfix in phosphate-buffered 1% OsO_4 . After further rinsing in three changes of distilled water for 30 min, the tissue was stained en bloc with 2% (wt/vol) uranyl acetate for 30 min at 60°C . After en bloc staining, the tissue was rinsed in three more changes of distilled water, dehydrated

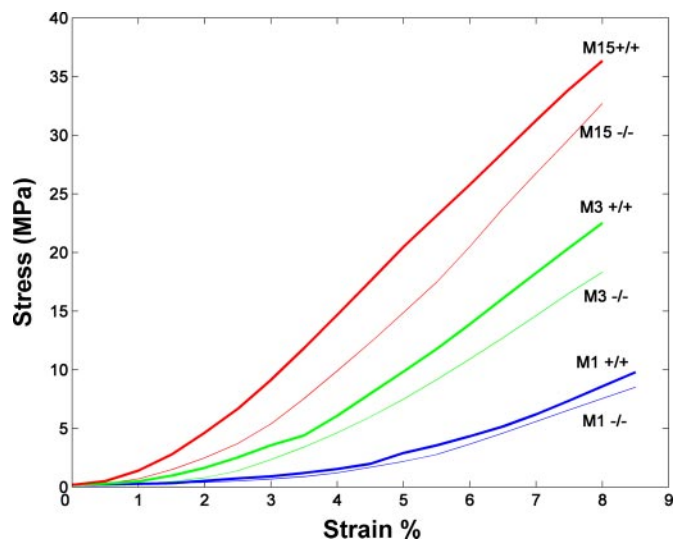


Fig. 1. Average curves of the ramp stretch for the 6 groups of the wild-type (+/+) and TIEG inducible early gene-1 (TIEG) (-/-) fascicles aged 1 mo (M1), 3 mo (M3), and 15 mo (M15) (M1^{+/+}, $n = 4$; M1^{-/-}, $n = 4$; M3^{+/+}, $n = 5$; M3^{-/-}, $n = 5$; M15^{+/+}, $n = 4$; and M15^{-/-}, $n = 3$).

in progressive concentrations of ethanol and 100% propylene oxide, and embedded in Spurr's resin (13). Thin sections (0.75 μm) were placed on a slide and stained with 1% (vol/vol) toluidine blue to identify the fascicles. After having selected the fascicle area, thin (90 nm) sections of the selected area were cut on a Reichert Ultracut E ultramicrotome (Bannockburn, IL), placed on 200 mesh copper grids, and stained with lead citrate. Five fascicles per tendon were selected. Four micrographs showing the representative structure of the fibrils were taken for each fascicle. The 20 transmission electron microscopy (TEM) pictures per tendon were obtained with a JEOL 1200 EXII TEM operating at 80 kV with a magnification of $\times 42,000$. Image analysis software (QWIN, Leica) was used to quantitatively analyze the ultrastructure of the wild-type and TIEG^{-/-} tendon fascicles. The average fibril diameter, fibril diameter distribution, and number of fibrils per unit area were measured.

Statistical Analysis

Each of the above-mentioned mechanical tests were performed on the four to five fascicles that were isolated from each wild-type and TIEG^{-/-} mouse. The values resulting from the four to five fascicles were averaged for each mouse, and this averaged value was representative of that individual. Therefore, each mouse group was composed of three to five individual values for each mechanical test. A two-factor (mouse type, age) ANOVA test was performed on these averaged values with the software Statgraphics 5.0 (Sigma Plus). The effects of group, the effects of age, and the interactions between group and age on stresses (σ_f and σ_s), viscoelastic stress relaxation, E , and fibril diameter were determined. When F values were significant, post hoc t -tests (Student-Newman-Keuls) were performed.

RESULTS

Effect of Age on the Mechanical Properties of Single Tail Tendon Fascicles

Figure 1 illustrates the behavior of the E from the ramp stretch test performed on the wild-type and TIEG^{-/-} fascicles. The E increased significantly ($P < 0.05$) with age from 1 mo ($E = 205.67 \pm 30.49$ MPa) to 15 mo ($E = 575.51 \pm 47.69$ MPa) for the TIEG^{-/-} mice, whereas the wild-type +/+ mice showed only a significant increase ($P < 0.05$) between 1 mo

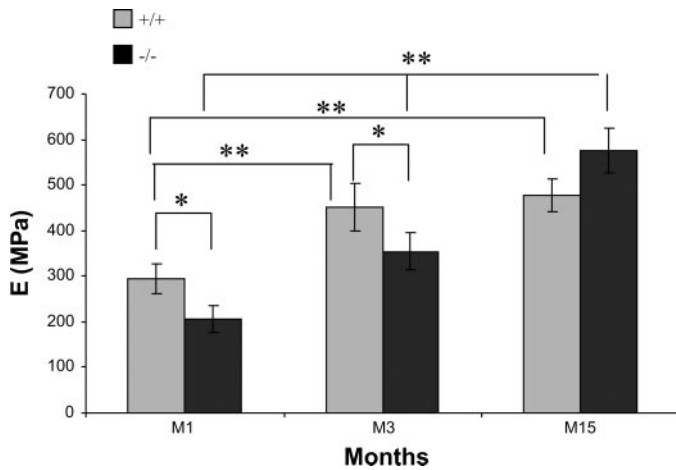


Fig. 2. Average values \pm SE) of the Young's modulus (E) for the wild-type (+/+) and TIEG^{-/-} fascicles aged 1 mo (M1), 3 mo (M3), and 15 mo (M15). ** $P < 0.05$, * $P < 0.1$.

($E = 293.12 \pm 32.66$ MPa) and 3 mo ($E = 450.58 \pm 52.92$ MPa) (Fig. 2). Between 1 and 3 mo, the E increased with the same ratio for wild-type and TIEG^{-/-} mice. Between 3 and 15 mo, the E increased $\sim 5.5\%$ for the wild-type mice, whereas a greater increase (38.4%) was measured for the TIEG^{-/-} mice (Fig. 2).

Figure 3 summarizes σ_f and σ_s measured during the relaxation test for the wild-type and TIEG^{-/-} fascicles. The measurement of σ_s showed a significant increase ($P < 0.05$) with age from 1 mo ($\sigma_s^{+/+} = 6.45 \pm 0.15$ MPa, $\sigma_s^{-/-} = 5.74 \pm 0.70$ MPa) to 15 mo ($\sigma_s^{+/+} = 27.84 \pm 1.78$ MPa, $\sigma_s^{-/-} = 33.64 \pm 4.59$ MPa) for the wild-type (+/+) and TIEG^{-/-} fascicle groups (Fig. 4A). The σ_f followed the same behavior as that of the σ_s for the TIEG^{-/-} mice, whereas the wild-type mice showed only a significant increase ($P < 0.05$) between 1 mo ($\sigma_f^{+/+} = 16.36 \pm 1.16$ MPa) and 3 mo ($\sigma_f^{+/+} = 37.38 \pm 1.65$ MPa) (Fig. 4B). The viscoelastic stress relaxation showed

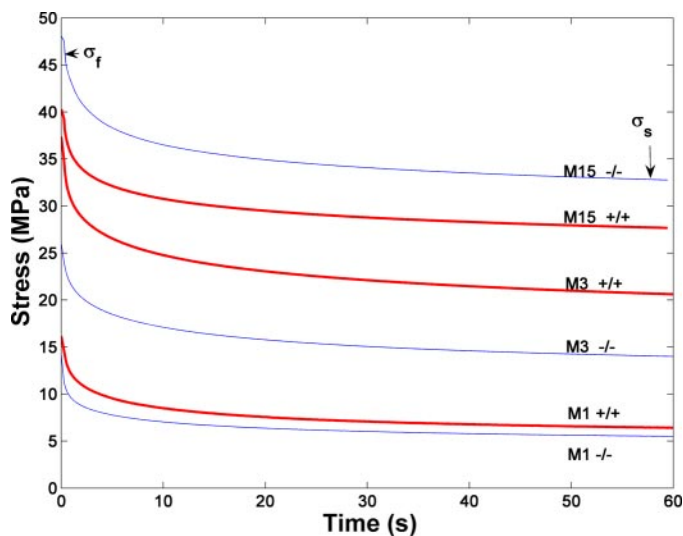


Fig. 3. Average curves of the stress relaxation for the 6 groups (M1^{+/+}, $n = 4$; M1^{-/-}, $n = 4$; M3^{+/+}, $n = 5$; M3^{-/-}, $n = 5$; M15^{+/+}, $n = 4$; M15^{-/-}, $n = 3$). σ_f , Fast stress; σ_s , static stress.

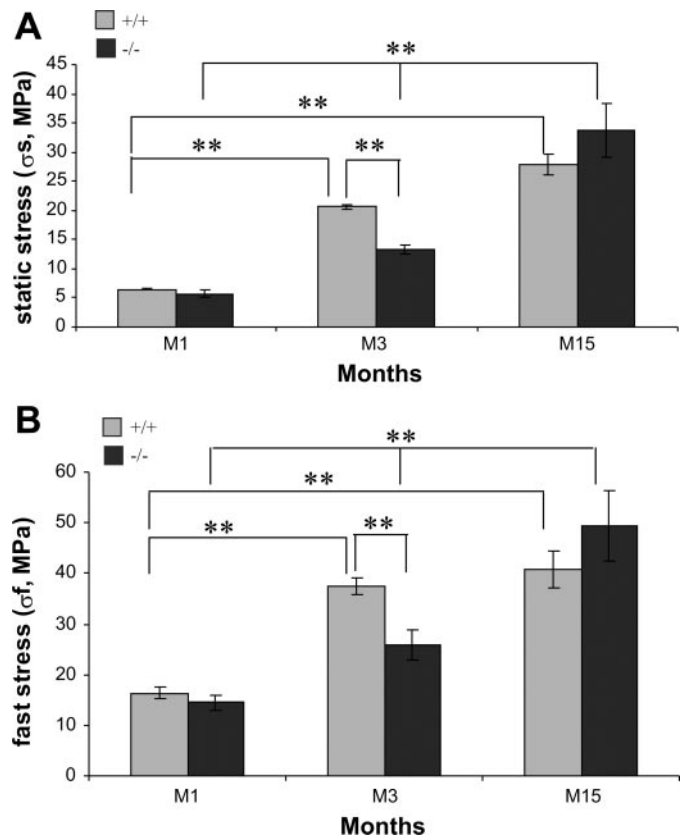


Fig. 4. Average values \pm SE of σ_f (A) and σ_s (B) for the wild-type (+/+) and TIEG^{-/-} fascicles. ** $P < 0.05$, statistical analysis of the mechanical parameters between groups.

a significant ($P < 0.05$) decrease with age for both wild-type and TIEG^{-/-} mice (Fig. 5).

Between 1 and 3 mo, the increase in the mechanical parameters for the wild-type mice ($\Delta\sigma_f = 21.02$ MPa, $\Delta\sigma_s = 14.16$ MPa) is about twice as much as that from the TIEG^{-/-} mice ($\Delta\sigma_f = 11.32$ MPa, $\Delta\sigma_s = 7.61$ MPa). Between 3 and 15 mo, σ_f and σ_s showed the opposite phenomenon with a much greater difference for the TIEG^{-/-} mice ($\Delta\sigma_f = 23.54$ MPa, $\Delta\sigma_s = 20.30$ MPa) compared with the wild-type mice ($\Delta\sigma_f = 3.43$ MPa, $\Delta\sigma_s = 7.23$ MPa) (Fig. 4, A and B).

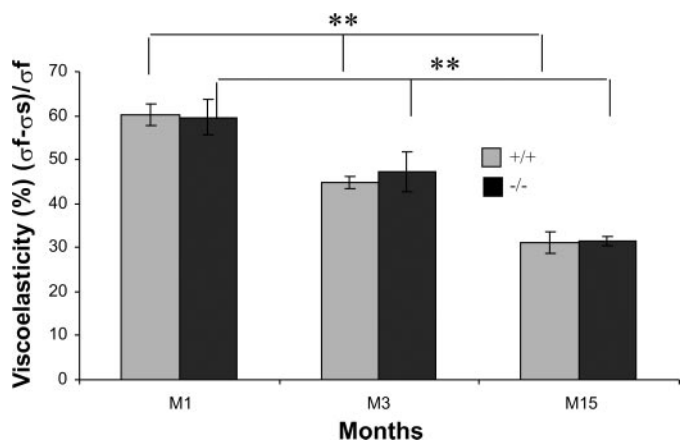


Fig. 5. Average percentage \pm SE of viscoelastic stress relaxation for the wild-type (+/+) and TIEG^{-/-} fascicles. ** $P < 0.05$.

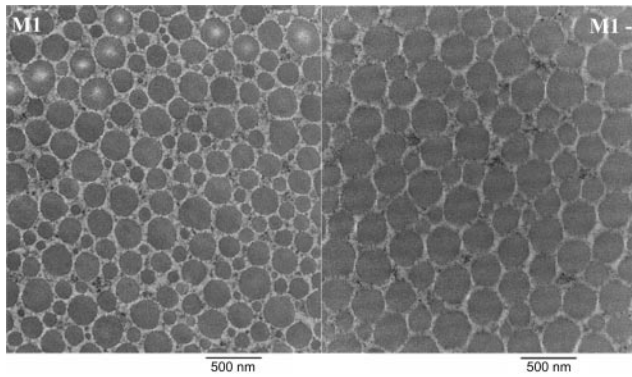


Fig. 6. Representative fascicle structure found in 1-mo animals ($M1^{+/+}$ and $M1^{-/-}$) with transmission electron microscope (TEM; $\times 42,000$ magnification).

Effect of Genotype on the Mechanical Properties of Single Tail Tendon Fascicles

At 1 mo of age, σ_f and σ_s as well as viscoelastic relaxation revealed no significant difference between the wild-type and the $TIEG^{-/-}$ mice (Figs. 4, A and B, and 5). However, the E showed a significant decrease in this age group ($P < 0.1$) (Fig. 2).

At 3 mo of age, all the mechanical parameters (σ_f , σ_s , and E), except the viscoelastic relaxation, showed a significant difference ($P < 0.05$, $P < 0.1$) between the wild-type and the $TIEG^{-/-}$ mice (Figs. 2, 4, A and B, and 5).

At 15 mo of age, all the mechanical parameters showed no significant difference between the wild-type and the $TIEG^{-/-}$ mice (Figs. 2, 4, A and B, and 5).

TEM

Figures 6–8 were obtained with a TEM (magnification $\times 42,000$) and show the representative structure of the different tail tendon fascicles (wild type and $TIEG^{-/-}$) aged 1, 3, and 15 mo. The average fibril diameter (D) measured for the $TIEG^{-/-}$ mice at 1 and 3 mo is larger ($D_{M1}^{-/-} = 194.3 \pm 73.4$ nm, and $D_{M3}^{-/-} = 233.6 \pm 86.4$ nm) than the diameter of the wild-type groups aged 1 and 3 mo ($D_{M1}^{+/+} = 146.7 \pm 86$ nm, and $D_{M3}^{+/+} = 209.3 \pm 85$ nm) (Figs. 6 and 7). However, at 15 mo, the fibril diameters were similar between the wild-type and $TIEG^{-/-}$ mice ($D_{M15}^{+/+} = 255 \pm 96$ nm, and $D_{M15}^{-/-} = 245 \pm 99$ nm) (Fig. 8).

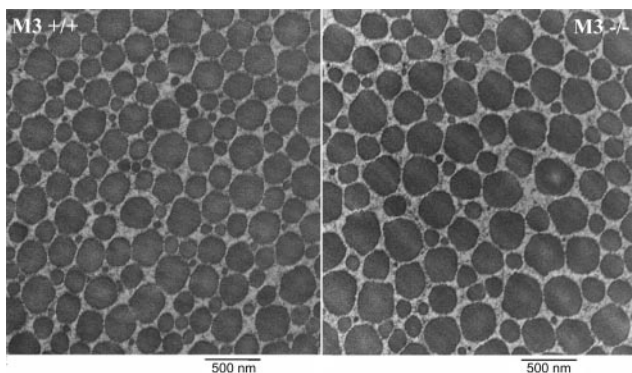


Fig. 7. Representative fascicle structure found in 3-mo animals ($M3^{+/+}$ and $M3^{-/-}$) with TEM ($\times 42,000$ magnification).

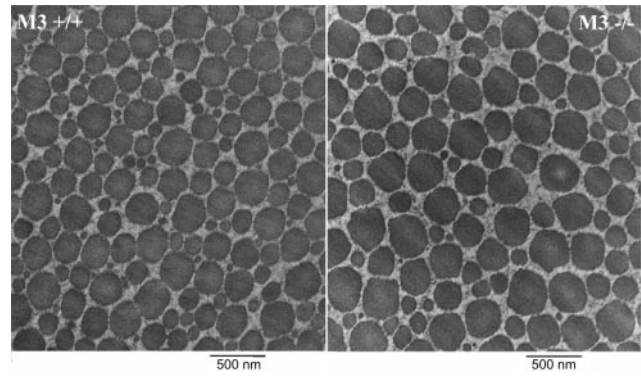


Fig. 8. Representative fascicle structure found in 15-mo animals ($M15^{+/+}$ and $M15^{-/-}$) with TEM ($\times 42,000$ magnification).

At 1 mo, the wild-type and $TIEG^{-/-}$ fascicles showed a mixture of large and small fibrils, measuring between 50 and 250 nm in diameter (Fig. 9). The $TIEG^{-/-}$ mice had a significantly ($P < 0.05$) higher percentage (20.61 vs. 16.31%) of fibrils with a diameter between 50 and 150 nm, whereas the wild-type mice had more fibrils (46.4 vs. 41.2%) with a diameter between 150 and 250 nm ($P < 0.05$) (Fig. 9). Very few fibrils with a diameter lower than 50 nm were found in the 1-mo $TIEG^{-/-}$ group, whereas the wild-type group had a considerable proportion of these smaller fascicles (17.5%) (Fig. 9). At the opposite end of the spectrum, the $TIEG^{-/-}$ mice showed an important presence (38.1%) ($P < 0.05$) of fibril diameters between 250 and 400 nm compared with the wild-type group, with only 19.7% of fibrils in this range (Fig. 9).

At 3 mo, the fibril diameter distribution can be divided into three parts. Fibrils with a diameter between 50 and 200 nm were more common ($P < 0.05$) (38.6%) in the wild-type group than in the $TIEG^{-/-}$ (31.5%) mice (Fig. 10). The proportion of fibril diameters between 200 and 350 nm was similar between the wild-type (52.1%) and $TIEG^{-/-}$ (53%) mice (Fig. 10).

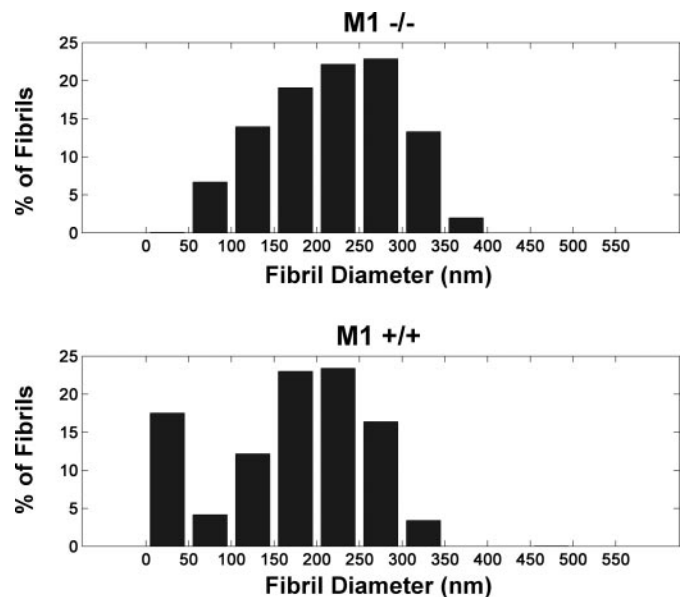


Fig. 9. Percentage of fibril distribution as a function of the fibril diameter (nm) for the 1-mo wild-type ($M1^{+/+}$) and $TIEG^{-/-}$ ($M1^{-/-}$) groups.

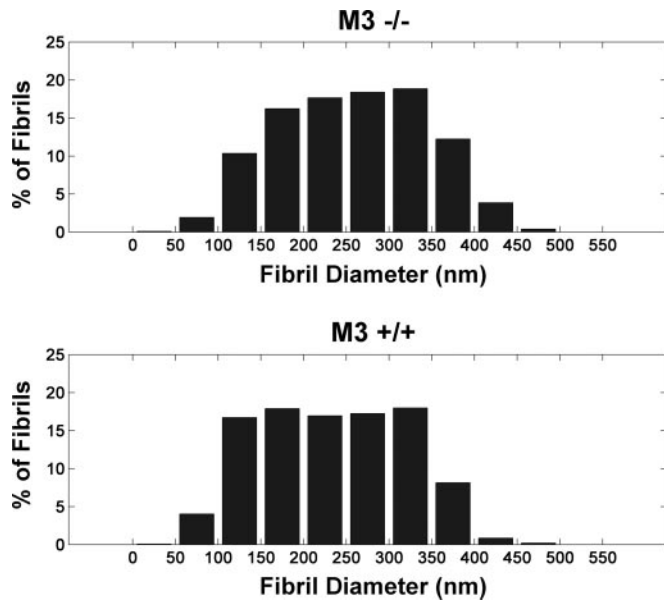


Fig. 10. Percentage of fibril distribution as a function of the fibril diameter (nm) for the 3-mo wild-type ($M3^{+/+}$) and TIEG $^{-/-}$ ($M3^{-/-}$) groups.

Finally, there was a higher percentage ($P < 0.05$) of fibril diameters between 350 and 500 nm for the TIEG $^{-/-}$ (15.5%) mice compared with the wild-type (9.2%) mice (Fig. 10).

At 15 mo, the distribution of fibril diameters looks like a Gaussian bell curve (Fig. 11). Fibril diameters between 50 and 300 nm were more common in the TIEG $^{-/-}$ (63%) mice than in the wild-type (54.1%) mice, with a corresponding decrease ($P < 0.05$) in the proportion of fibril diameters from 300 to 550 nm for the TIEG $^{-/-}$ mice (37 vs. 45.9%) (Fig. 11).

DISCUSSION

It has been well established that TGF- β is an important factor affecting tendon healing (11) and tendon strength (1, 9). To further elucidate the mechanisms of TGF- β action in tendons, it is important to understand the role of specific genes responsible for mediating the actions of TGF- β in this tissue. Based on the identification of TIEG as a TGF- β inducible early gene and the generation of TIEG knockout, it was of interest to determine whether TIEG plays an important role in the microarchitecture and mechanical properties of tendons. The results of these studies reveal that the tail tendons of TIEG $^{-/-}$ mice are significantly less stiff than the wild-type controls at 3 mo of age. Interestingly, no difference existed at 1 or 15 mo, indicating that they are age-dependent changes in the mechanical properties of tendon in TIEG $^{-/-}$ mice. In addition, the ultrastructure of the tendons (i.e., fibril size) exhibited significant differences between wild-type and TIEG $^{-/-}$ mice at 1 and 3 mo of age. Taken together, these data imply an important age-dependant role for TIEG in the growth and maintenance of tendon microarchitecture and strength in adult mice.

To fully understand the biological mechanisms behind the development, growth, and remodeling of connective tissues, including tendons, it is necessary to identify specific genes and their contribution to these processes. Yamamoto et al. (18) showed that there is a strong correlation between ground substance material and the mechanical properties of collagen fascicles. The effects of static (19) and cyclic (21) stresses on

the mechanical properties of cultured collagen fascicles have also been analyzed, and the results reveal a relationship between mechanical and structural properties on one hand and modification of the static and cyclic tensile stresses on the other. Other investigations have been performed that demonstrate that other elements may influence the mechanical properties of collagen fascicles and tendons. A transgenic mouse model, in which no procollagen α_1 is synthesized (Mov13 transgenic mice), was used to determine the relationship between composite structure and mechanical properties of tendon (4, 5). These studies revealed that a moderate correlation between the morphological properties (fibril diameter) and both stiffness ($r = 0.73$) and maximum load to failure ($r = 0.75$) exists, whereas a weak correlation exists between the morphological properties (fibril diameter) and the modulus ($r = 0.39$) with the maximum stress ($r = 0.38$) (4, 5). The structure-function relationship was thus again established. Moreover, analysis of the proteoglycans and glycosaminoglycans reveals that the composition of matrix molecules influences the structure-function relationship of tendon (4, 5).

In general, the mechanical parameters measured in the present study are in agreement with those obtained by Derwin et al. (4), taking into consideration the slight differences in age and stretch parameters. Moreover, the viscoelastic behavior observed during the relaxation test in the present report was also demonstrated in another study (6). To examine the viscoelastic behavior of tail tendon fascicles, the holding time used by other studies (6) was much greater (600 s) than those used in our investigation. However, no differences were detected in our study between 60 and 180 s (data not shown). In addition, the purpose of our study was not to analyze the viscoelastic behavior but rather to investigate the possible role of TIEG in the mechanical properties of the tendon fascicles. The effects of TIEG were mainly observed in adult mice (3 mo) with a significant difference in the mechanical parameters between the wild-type and TIEG $^{-/-}$ mice individuals. In parallel with the mechanical properties, the morphological

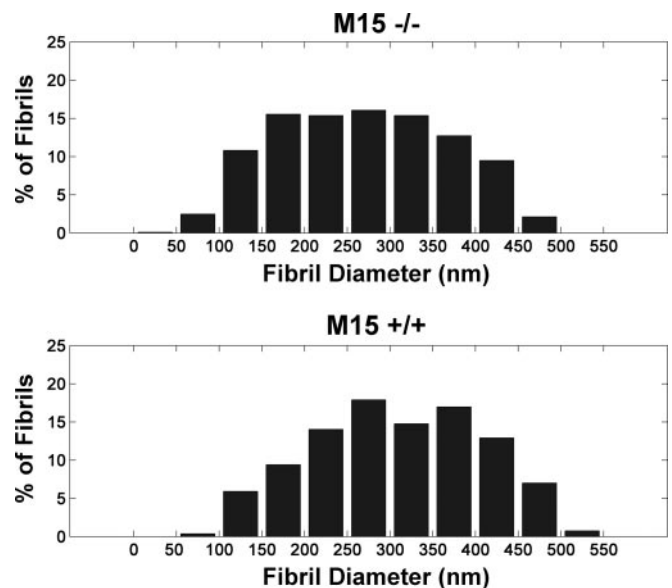


Fig. 11. Percentage of fibril distribution as a function of the fibril diameter (nm) for the 15-mo wild-type ($M15^{+/+}$) and TIEG $^{-/-}$ ($M15^{-/-}$) groups.

analysis showed a larger fibril size for the adolescent and adult TIEG^{-/-} mice compared with wild-type controls. However, the larger fibrils found in TIEG^{-/-} mice aged 1 and 3 mo did not lead to an increase in mechanical properties. This suggests that the effect of TIEG on fibril mechanical properties is not related simply to an effect on fibril diameter but may have to do with cross-linking. Indeed, the larger fibril diameter in TIEG^{-/-} mice may represent an attempt to compensate for a reduction in the concentration or stability of collagen cross-links. It is also possible that differences in the mechanical properties and microarchitecture were not observed at each age group examined due to compensation for loss of TIEG expression by other highly related genes. Future studies should examine the effect of TIEG on the expression and production of proteins important for tendon structure and function, including collagen and proteoglycans.

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