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Ultrastructural Determinants of Murine Achilles Tendon Strength During Healing

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The mechanisms by which tendon strength is established during growth and development and restored following injury are not completely understood and are likely to be complex, multifactorial processes. Several studies examining the relationship between mechanical behavior and ultrastructural characteristics of tendons and ligaments during growth and maturation suggest that collagen fibril diameter is strongly correlated with tendon strength. Because of the similarities between development and repair processes of musculoskeletal tissues, increases in tendon strength during healing may be related to increases in fibril ultrastructural parameters such as fibril size, numerical density, and area fraction. In this study, we compared murine Achilles tendons at various time points after tenotomy with sham-operated controls in tensile tests to failure and examined tendons using electron microscopy to assess collagen fibril ultrastructure. We found that in the 6-week period following Achilles tenotomy, fibril mean diameter remained significantly smaller than sham-side diameter by a factor of 2–3. Despite the persistently small fibril size, increasing numerical density resulted in a gradual increase in fibril area fraction. Biomechanical strength did not reach that of intact tendons until some time between 5 and 7 weeks, approximately the same time period when fibril area fraction began to approach sham values. These data suggest that parameters other than collagen fibril size are most responsible for increased tendon strength during healing.

Keywords Collagen, Fibrils, Healing, Tendon.

INTRODUCTION

The establishment of tendon strength during development and growth, as well as its restoration following injury, are poorly understood processes but are likely to be complex and multifactorial. From a structural standpoint, the load to failure that a tendon can sustain is influenced by both the overall geometry of the tendon (most important, its cross-sectional area), as well as its intrinsic material properties. As type I collagen is the major constituent of the extracellular matrix of tendon (comprising over 85% of its fat-free dry weight), characteristics related to collagen such as total collagen content; such microarchitectural features as the arrangement of individual collagen molecules into microfibrils, subfibrils, fibrils, and fascicles; and ultrastructural features such as collagen fibril size, area fraction, and numerical density are all likely to be important determinants of tendon strength. In addition, the amount and relative distribution of various proteoglycans and glycoproteins and the degree of hydration and collagen cross-linking are also important variables. What remains less clear is the relative importance of these various factors in determining tendon mechanical behavior, particularly during repair.

The relationship between the mechanical behavior of tendons and the ultrastructural characteristics of collagen fibrils within the tissue has been extensively studied during growth and maturation processes [1–3]. Several studies suggest that collagen fibril diameter is strongly and positively correlated with tendon strength [1, 4]. In mouse- and rat-tail tendons, collagen fibril diameter markedly increases from birth to maturity, with the largest changes occurring from a few weeks to a few months after conception [1–3]. Concomitant with these changes are significant increases in tail tendon strength [1, 4]. The viscoelastic behavior of tendons also appears to change during this same period [5].

An association between fibril diameter and tissue strength also occurs in other collagenous tissues. Differences in strength between rabbit medial collateral ligaments and anterior cruciate ligaments correlate with differences in fibril diameter [6]. Skin also appears to conform to this relationship, as skin fibrils from

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animals with more active skin tensile requirements (e.g., fish, amphibians) have a bimodal or right-skewed size distribution when compared with fibrils from birds or mammals (i.e., the larger the tensile requirements, the larger the fibrils and greater the distribution) [7, 8].

Because development and repair of tendon are both processes during which extracellular matrix constituents are rapidly synthesized and remodeled in an effort to establish mechanical integrity, increases in tendon strength during healing may be related to increases in fibril ultrastructural parameters. To our knowledge, however, no detailed time-course investigation has been performed to date in which both biomechanical and ultrastructural data have been examined at multiple time points during the healing process in an attempt to correlate the two. The objective of our study, therefore, was to test the hypothesis that increases in tendon strength during healing can be explained by concomitant increases in collagen fibril ultrastructural parameters over a period of 6 weeks.

METHODS

Operative Technique

Midsubstance tenotomies were performed on the left Achilles tendon of 8-week-old male A/J strain mice (mean body mass ± SD at the time of surgery was 18.87 ± 1.85 g). The right Achilles served as a sham-operated control. All experimental protocols were approved by the appropriate institutional animal care and use committee. Surgical procedures were performed with standard aseptic techniques, using a 50 μL intramuscular injection of 37.5% ketamine and 12.5% xylazine in sterile saline for anesthesia. A second bolus injection of 25 μL was administered as needed.

On the left (operative) side, a vertical incision approximately 1 cm in length was made in the posterior midline just proximal to the calcaneus using a No. 15 blade scalpel. The Achilles tendon was carefully freed from the underlying tissue and isolated with a flat spatula placed between the tendon and adjacent bone. A 6-0 blue Surgipro suture (SP-1697, Owens & Minor, Richmond, VA, USA) was then passed twice through the proximal portion of the tendon in a modified Kessler technique, ~3 mm distal to the musculotendinous junction. A similar stitch also was passed through the distal tendon, ~3 mm proximal to the calcaneal insertion. Once both sutures were in place, the tenotomy was created between the two sutures with the scalpel, then repaired by tying the free ends of the sutures. Gentle freehand tension was used to test the repair, and the sutures resecured if any slippage was noted.

The wound was copiously irrigated with sterile saline solution and the skin closed with three or four interrupted 6-0 nylon sutures (SN-1696, Owens & Minor). On the right (sham) side, an identical procedure was followed to separate the tendon from underlying bone and surrounding soft tissue, but no tenotomy was performed and no sutures were introduced. The tissue was irrigated and the skin closed in a manner identical to the contralateral side.

After surgery, the mice recovered in a temperature-controlled environment to prevent hypothermia, then returned to their cages where they received water and food *ad libitum* and were allowed full activity. At the appropriate time points, the mice were weighed and sacrificed via CO_2 inhalation. Immediately after sacrifice the hind limbs were dissected at the hip joint. Samples used for electron microscopy were immediately placed in fixative using the protocol to be described under "Ultrastructural Analyses." Samples used for mechanical testing were thoroughly moistened with phosphate buffered saline (PBS), wrapped in saline-soaked gauzed, and stored at -20°C in hermetically sealed bags until further analysis.

Biomechanical Analyses

Biomechanical analyses were performed on tendons from 31 mice at 7, 14, 21, 35, 49, and 63 days after tenotomy, with an additional basal control group (no surgery) at time zero ($n = 5$ for days 7, 14, and 49; $n = 4$ for all others). Immediately prior to testing, individual limbs were thawed in PBS at room temperature for 1 hr. The tendons were harvested through an anterior incision extending from the midthigh to the foot to avoid any inadvertent destruction of the underlying tissue that might be caused by a posterior incision. The skin was circumferentially removed from the limb, and the Achilles tendon was separated from the underlying bone and freed with a transverse cut across the middle of the muscle belly at the proximal end of the tendon and with a cut through the calcaneus at the distal end. Any remaining adherent soft tissue was carefully removed. In addition, the gastrocnemius fibers were bluntly dissected and removed, leaving only the intramuscular tendon fibers. In all operative-side specimens, including the 7-day group, we observed that the suture had pulled out and no longer bridged the tenotomy site. To avoid unnecessary tissue damage, no attempt was made to remove the suture remnant. The tendon was then prepared for testing using previously published methods from our laboratory [9, 10].

The construct was mounted on an Instron Mechanical Testing Machine (Model 5542, Instron Corp., Canton, MA, USA) and the tendon preloaded to 0.02% body weight. Tendon gauge length was measured with precision sliding calipers, and the tendons loaded to failure at 100% strain (change in length/gauge length) per second. This strain rate was chosen as it is representative of physiologic rates of loading experienced in vivo [11, 12]. Force versus extension data were sampled at a frequency of 20 Hz and the following four parameters calculated for each tendon: maximum load to failure (i.e., structural strength), strain at maximum load, structural stiffness (slope of the force versus extension curve), and energy absorbed to failure (area under the force versus extension curve). For each animal, all data for the operated tendon were normalized to sham-side data prior to statistical analyses. Tendons were inspected after testing to determine mode of failure.

Ultrastructural Analysis

Healing and sham-operated Achilles from 7 representative mice at 3, 5, 7, 12, 14, 28, and 42 days after tenotomy were

TABLE 1
Failure mode of mechanically-tested tendons.*

	Operated side (left)			Sham side (right)		
Days post-op	Avulsion (%)	Calcaneal insertion (%)	Mid-substance (%)	Avulsion (%)	Calcaneal insertion (%)	Mid-substance (%)
0 (control)	100	—	—	100	—	—
7	—	—	100	100	—	—
14	—	—	100	60	20	20
21	—	—	100	100	—	—
35	—	25	75	50	25	25
49	—	40	60	80	20	—
63	25	25	50	75	—	25

*Numbers represent percentage of failures that occurred via a given mode for each group.

processed for transmission electron microscopic (TEM) analysis of collagen fibril morphology. Tendons were harvested in an identical manner to that described above. The muscle-tendon-bone unit was placed in 2.5% glutaraldehyde with 0.1 M sodium cacodylate buffer at a pH of 7.4 for 3 days immediately following sacrifice. Further dissection was used to obtain a cylinder of tissue approximately 1 mm in length from the center of each tendon. These samples were placed overnight in 1% (weight per volume) OsO4 in 0.1 M sodium cacodylate buffer, pH 7.4 at room temperature. They were then dehydrated through graded ethanol solutions after four rinses of 5 min each in 0.1 M cacodylate buffer, pH 7.4. Infiltration in Epon plastic was performed with four changes of 15 min each in propylene oxide (PPO) followed by 24 hr of PPO:Epon at 3:1, 24 hr at 1:1, 72 hr at 1:3, and 24 hr in 100% Epon. Specimens were then allowed to polymerize for 7 days at 60°C.

Transverse sections of the tendons approximately 70 μm thick were cut with an Autocut ultramicrotome (Leica Microsystems, Vienna, Austria), constrasted with lead-citrate and uranyl-acetate, and imaged with a transmission electron microscope (Hitachi-7100B, Tokyo, Japan). Using random sampling methods [13], 11 photos per tendon were taken at a magnification of 80,000× and digitally scanned. All individual fibrils within each photo were outlined and analyzed using standard imaging software (ImagePro Plus, Media Cybernetics, Silver Spring, MD, USA). The following three parameters were determined for each tendon: mean fibril diameter, fibril number per unit area (number of fibrils per mm^2), and fibril area fraction (percentage of each photo area occupied by fibrils). Individual fibril diameters were measured using the minimum diameter values to avoid any error induced by fibrils cut obliquely to the long axis. The Gundersen method was used to determine individual fibril counts within each photograph [13]. Approximately 2,500 fibrils per animal per time point were analyzed.

Data Analysis

All dependent biomechanical and ultrastructural variables were analyzed using a one-factor ANOVA and Fisher's projected least significant difference post-hoc analyses with healing time as the independent variable. A cutoff level of $p \leq .05$ was chosen for statistical significance.

RESULTS

Biomechanics

The vast majority of sham-operated tendons failed via avulsion, whereas operated tendons failed predominantly at the tendon midsubstance in the first few weeks following surgery with a gradual increase in frequency of failures at the bone/tendon junction and via avulsion as healing time progressed (Table 1). Raw biomechanical data for operated-side tendons are shown in Table 2, while normalized results (healing/sham side) are provided in Figures 1A–D. Maximum normalized structural strength values of the operated tendons averaged only 14.4% at 1 week post-tenotomy; this difference was significant compared with the control group. Strength values increased steadily after surgery but remained significantly different from the controls through week 5, after which values became statistically comparable with the basal (time zero) values (Figure 1A). Strain at maximum load demonstrated no appreciable pattern of statistical significance during the period of testing (Figure 1B). Structural stiffness followed a course similar to maximum load, with results remaining significantly lower than controls throughout the 6-week period, but with a significant increase occurring between days 21 and 35 (Figure 1C). Energy absorbed to failure demonstrated a significant increase compared with controls during days 21 through 49, followed by a return to values comparable with controls (Figure 1D).

Ultrastructure

Collagen fibrils from healing tendons at all time points were significantly smaller than fibrils from intact, sham-operated tendons, and no significant changes in fibril diameter were detected between any of the healing groups over the 6-week period examined (Figures 2A and 3). By contrast, the number of fibrils per unit tendon area increased steadily over the time period,

TABLE 2
Raw biomechanical results of operated-side tendons (mean [SD]).

Days post-op	Maximum load (N)	Strain at maximum load (%)	Stiffness (N/mm)	Energy absorbed to failure (N-mm)
0 (control)	8.56 (2.47)	42 (21)	12.8 (7.4)	5.08 (2.78)
7	1.25 (0.41)*	86 (15)*	0.82 (0.40)*	2.69 (0.91)
14	2.30 (0.75)*	111 (44)*	1.45 (0.35)*	6.14 (3.41)
21	6.50 (0.65)†	70 (8)‡	3.43 (0.92)*	11.51 (2.82)*†
35	6.90 (1.46)†	62 (19)‡	3.80 (1.23)*	11.33 (2.11)*†
49	8.68 (1.51)†£	71 (19)‡	5.52 (1.41)*†	13.38 (4.08)*†
63	9.50 (2.25)†£§	73 (10)‡	5.67 (0.54)*†	13.64 (4.15)*†

*$p < .05$ vs. day 0 (control); †$p < .05$ vs. days 7 and 14; ‡$p < .05$ vs. day 14; £$p < .05$ vs. day 21; §$p < .05$ vs. day 35.

with a significant increase occurring between 12 and 14 days (Figure 2B). Because the fibrils in the healing tendons were so small at all time points, the increased fibril numerical density had a more gradual effect on the fibril area fraction (percentage of matrix area occupied by collagen fibrils) (Figure 2C). Although fibril area fraction was still significantly different from sham values by 6 weeks, the percentage of matrix occupied by fibrils increased steadily at a rate of approximately 5% (absolute) per week from 7 days post-op onward.

DISCUSSION

In this study, we examined the biomechanical performance and ultrastructural characteristics of murine Achilles tendons over a 6-week period following tenotomy. Although tendon strength and stiffness returned to levels comparable with controls by 5 to 7 weeks, collagen fibril mean diameter remained significantly smaller than sham-side diameter by a factor of 2–3 throughout this time period. Although the healing fibrils were smaller at all time points, we observed a steady increase in fibril

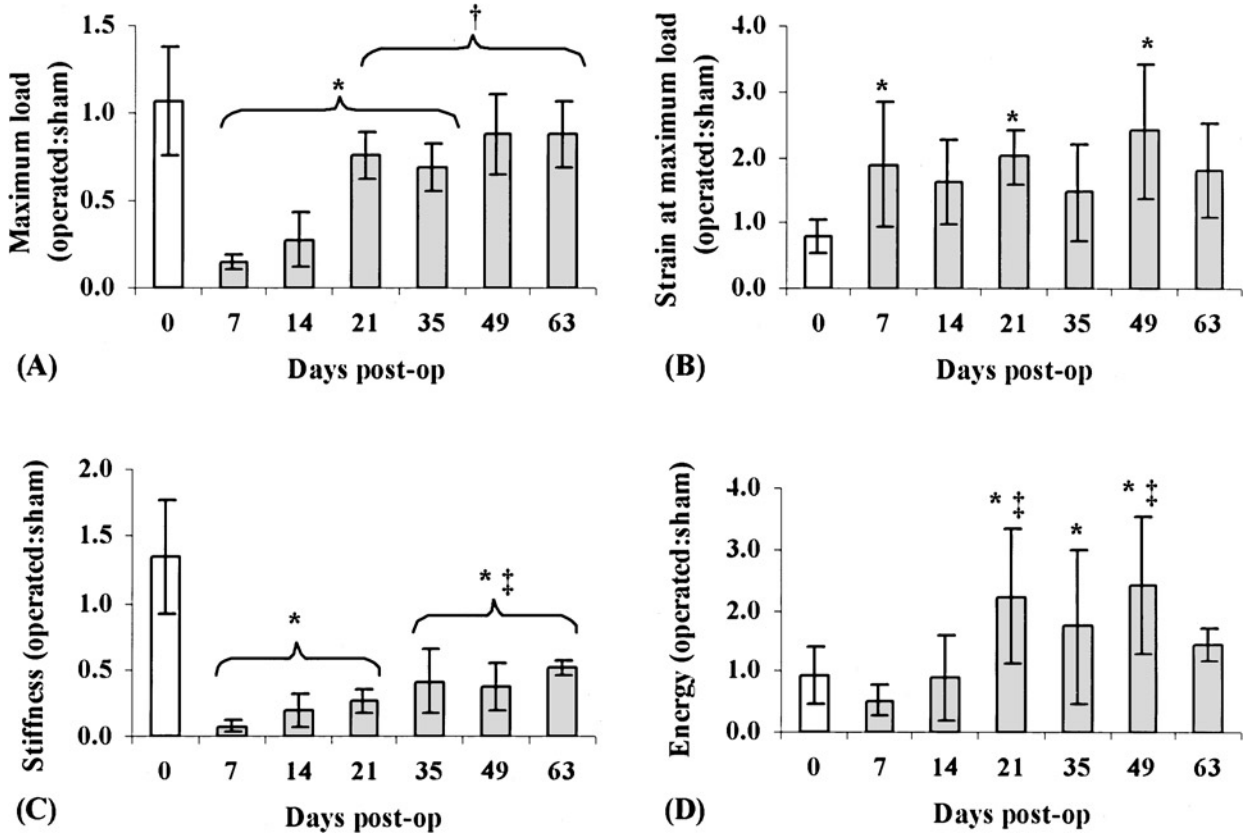

Figure 1. Biomechanical parameters of healing Achilles tendons as a function of time after tenotomy. (A) maximum load, (B) strain at maximum load, (C) structural stiffness, and (D) energy absorbed to failure. All operative side results are normalized to sham side data. $* = p < .05$ vs. group 0 (control group); $† = p < .05$ vs. days 7 and 14; $‡ = p < .05$ vs. day 7.

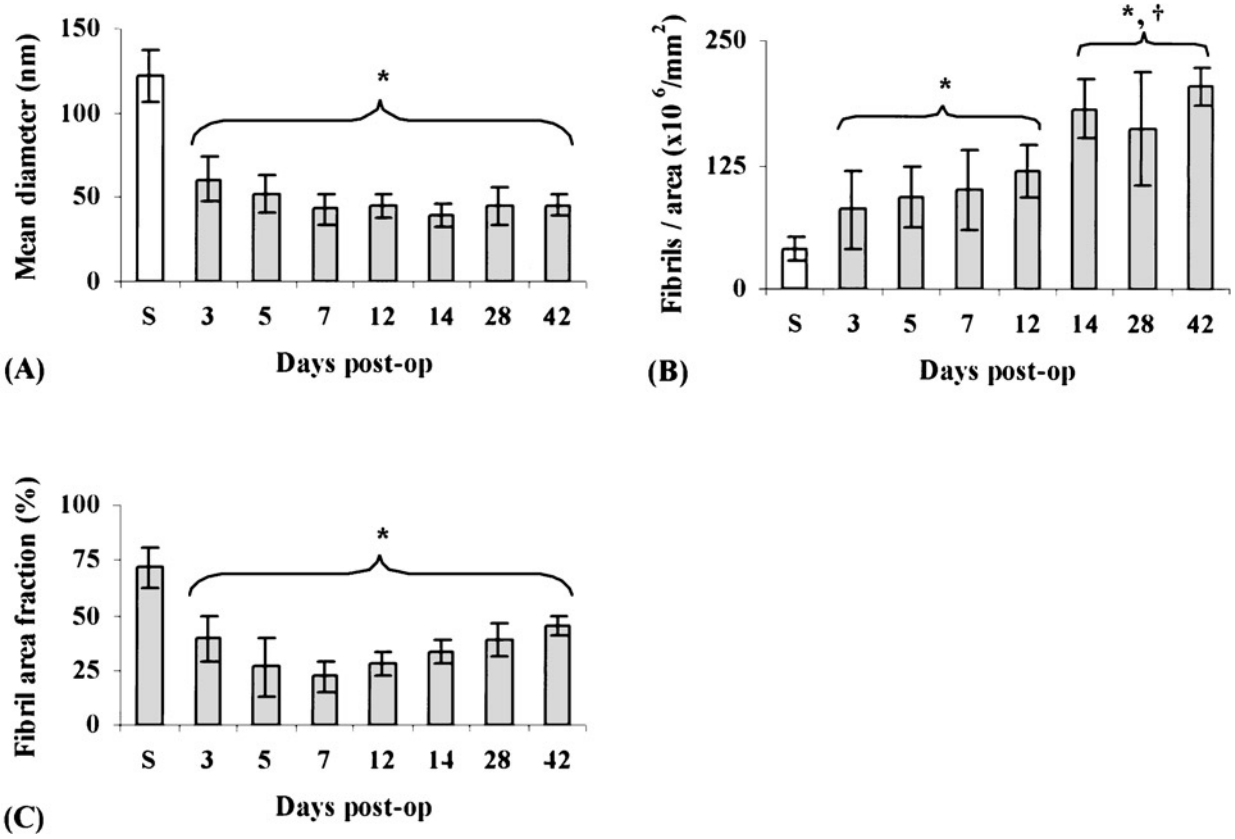

Figure 2. Ultrastructural parameters of healing Achilles tendons as a function of time after tenotomy. (A) mean diameter, (B) fibril number per unit area, and (C) fibril area fraction. S = sham-operated group; $* = p < .05$ vs. sham; $\dagger = p < .05$ vs. sham and days 3–12.

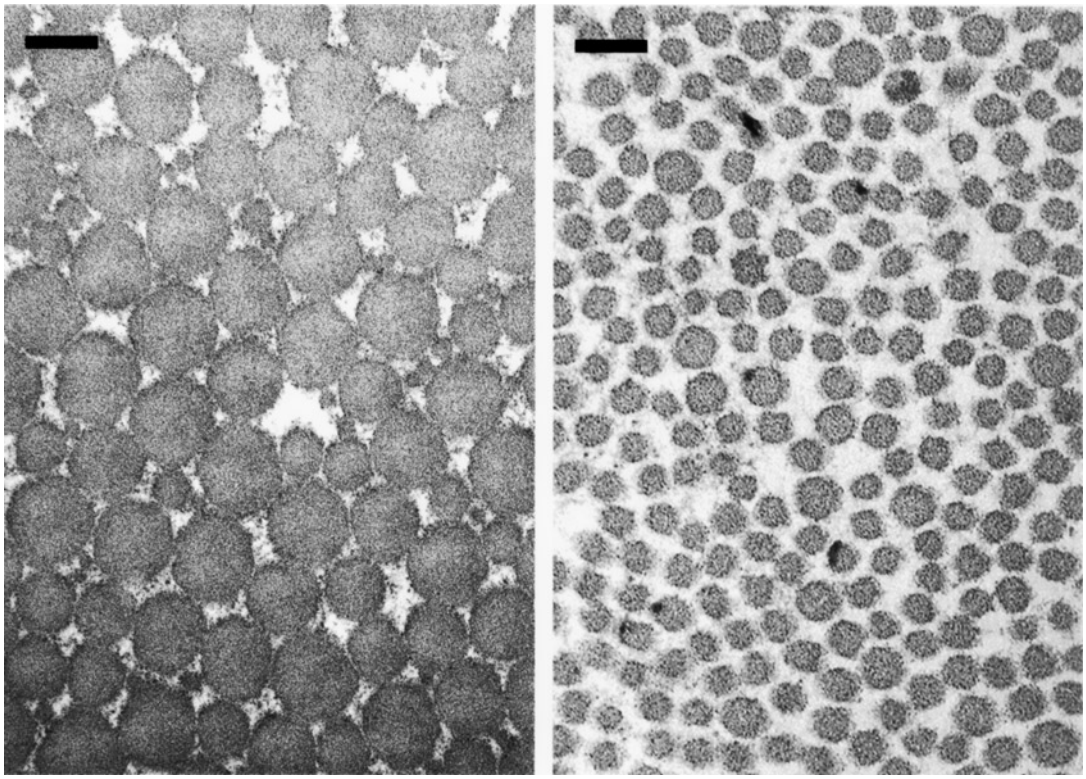

Figure 3. Representative cross-sections of sham (left) and healing (right) tendons at 42 days. Bar represents approximately 125 nm.

numerical density. The increasing numbers of fibrils in healing tendons resulted in a gradual increase in fibril area fraction, which steadily increased at 5% per week and would be predicted to reach sham values at approximately 9 weeks, the same time period when full biomechanical strength returned.

The time course for biomechanical recovery found here is consistent with prior studies of healing tendons. A recent study of Achilles tendon healing in rats noted a return to 60% of normal stiffness by 2–3 weeks [14]; studies of rabbit plantaris tendon described a return to near-normal strength and stiffness between 8 and 16 weeks post-tenotomy [15]. The healing time course appears to be fairly similar regardless of whether the tenotomy is managed nonoperatively or surgically repaired with any of a number of different suture methods [14–16]. Here, we noted that the suture had spontaneously failed in all operated-side specimens, as early as 1 week post-op. (The basal control group and all sham-side tendons had no suture introduced.) Although not removed for fear of causing unnecessary tissue damage, the suture should not therefore have altered the biomechanical properties of the tenotomy site. We chose to continue suturing the surgical defect, however, to approximate the tendon ends and provide some initial stability in the immediate postoperative period. It is likely the suture pulled out very soon after surgery, as the animals were allowed to ambulate freely immediately afterward.

Previous biomechanical studies of healing tendons from small animals often have had difficulties with tendon fixation during testing and were frequently complicated by slippage of the tendon from the clamp, failure at the tendon-clamp interface, or muscle belly failure when complete muscle-tendon complexes were used [17, 18]. Here, we used a previously published technique from our group [9, 10] and dissected the muscle fibers from the intramuscular tendon fibers, allowing us to secure the tendon ends firmly within the clamp. No slippage was noted in any tests based on visual inspection, and all tendons failed at the tenotomy site, calcaneal insertion, or via avulsion (i.e., no failures occurred due to the proximal portion of the tendon slipping out of the grips, Table 1).

We also used a strain rate of 100% per second to approximate in vivo strain rates, as this value lies in the range of real-life rates noted in prior experiments [11, 12]. Freezing the tendons prior to testing should have had minimal effect on our results, as analyses of fresh soft tissue versus tissue following freezing and storage have demonstrated no significant differences in most biomechanical parameters except for small differences in the first few cycles of the hysteresis curve as long as dehydration is avoided [14, 19–21].

It is important to note that this study evaluated only structural biomechanical properties of the tendons. It is exceedingly difficult to accurately determine material properties of mouse tendon from structural properties, as this requires determination of tendon geometry (cross-sectional area). Unfortunately, the size of mouse Achilles tendons limits accurate measurement of cross-sectional properties. Newer measurement techniques using laser micrometers have much greater accuracy than earlier methods [21], but these were not available in our laboratory. Still, we believe that after 2 weeks, the structural data reflect material properties, as we noted no gross qualitative differences in tendon size between the sham-operated and healing tendons after $\sim$2 weeks postoperatively.

Although investigators have charted histological and biomechanical parameters after tendon injury [20–23], none to date also has examined the ultrastructural characteristics of collagen fibrils during the healing period. Previous studies have described qualitative changes in collagen content and increases in the number of smaller fibrils following tendon injury [17, 26, 27]. But we could find none that quantitatively assessed fibril architecture at multiple time points during the healing process, most likely due to the intensive nature of the analyses involved. To the best of our knowledge, this is the first study to detail both ultrastructure and biomechanical recovery of healing tendons across multiple time points.

One limitation of this study is that the tendons from only one representative animal per time point were analyzed to determine ultrastructural characteristics. Despite testing a number of image analysis programs, we wre unable to find any with the ability to accurately discriminate fibril outlines from the background matrix and thereby autotrace their shapes. We were therefore required to manually trace all fibrils analyzed, a number greater than 40,000 fibrils, which ultimately limited our ability to analyze a large sample size. Although our results were consistent across the time period, development and use of a program that could automatically trace and accurately assess fibrils would allow a greater number of tendons at each time point to be studied and further increase accuracy. A further weakness is the fact that the time points examined for the biomechanical and ultrastructural analyses do not correspond on a one-to-one basis. However, the sufficient number of time points examined and the overlap provided between the times chosen for the two sets of analyses should not diminish our conclusions, although additional TEM analysis of tendons at 9 weeks post-op would strengthen the findings by demonstrating a return to normal fibril area fraction.

Ours is not the first study to question the relationship between fibril size and tendon (or ligament) strength. Bay, in 1993, found collagen fibril diameter to be a poor predictor of elastic modulus in sheep cruciate ligaments [28]. Recently, Derwin also found only a mild correlation between mean fibril diameter and tendon stiffness and maximum load, and an even weaker correlation with tendon material properties [1]. These studies, in addition to our own, suggest that of the ultrastructural parameters often used to characterize tendon, collagen fibril size may not be the most important predictor of tendon strength. Indeed, fibril area fraction (which is related to both fibril size and number and also is most closely related to collagen content) appears to be a more relevant ultrastructural determinant of tissue strength. It is important to emphasize, however, that ultrastructural characteristics are only one subset of a large range of factors that are all likely to influence the tissue-level strength of a tendon, including proteoglycan types and distributions, hydration, and the degree of cross-linking, among others [29].

CONCLUSION

This study offers further evidence that, among the commonly examined ultrastructural parameters used to characterize tendon, collagen fibril diameter may play a smaller deterministic role in re-establishing tendon structural strength during repair than previously hypothesized. During the 6-week time period following Achilles tendon tenotomy in mice, collagen fibril mean diameter remained significantly smaller than sham-side fibril diameter by a factor of 2–3. Despite the small fibril size, fibril area fraction began to gradually increase toward sham values over the course of 6 weeks. This was due primarily to a significant increase in the number of fibrils per unit area.

Structural strength did not reach that of intact tendons until some time between 5 and 7 weeks, roughly the same time period when fibril area fraction began to approach sham values. These data challenge the assumption that increases in collagen fibril size are a primary determinant of increased tendon strength during healing. We suggest that, among the ultrastructural parameters commonly examined as potential determinants of tendon strength, the related parameter of fibril area fraction may have a more significant effect on tendon mechanical properties than previously acknowledged.

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