Semitendinosus Regrowth

Biochemical, Ultrastructural, and Physiological Characterization of the Regenerate Tendon

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Background: Previous studies have suggested that hamstring tendons can regenerate following harvesting for anterior cruciate ligament reconstruction.

Hypothesis: This “neo-tendon” is a true, functional tendon, not scar tissue.

Study Design: Controlled laboratory study.

Methods: Semitendinosus tendons were harvested from 35 New Zealand white rabbits using a standard tendon stripper. The rabbits were sacrificed 9 to 12 months following the index procedure and thoroughly evaluated.

Results: Thirty-one rabbits were available at the time of sacrifice. The neo-tendon was present in 26 rabbits but was highly variable in size and location of its tibial insertion. Histologic and immunohistochemical staining confirmed that the regenerate tissue was indeed tendon with normal cellularity, organization, and immunolocalization of type I collagen. Electron microscopy showed regeneration of organized collagen tissue that simulated native tendon but with a smaller cross-sectional diameter. Functionally, the neo-tendon was able to transmit force across the musculotendinous junction but at a significantly slower rate than the opposite, control leg. Biomechanical properties of the neo-tendon were significantly less than the control side. Biochemical analysis revealed that the neo-tendons contained glycosaminoglycans and collagen, but levels were significantly lower than normal tendons.

Conclusions: Semitendinosus tendons regenerate with biologically reactive tendinous tissues in an animal model. This tissue has many of the characteristics of a normal tendon but appears to be inferior to the original musculotendinous unit at 9- to 12-month evaluation. Further characterization of the “lizard tail phenomenon” is still needed.

Clinical Relevance: Hamstring tendon regrowth may have a dramatic impact on postoperative function of patients who undergo anterior cruciate ligament reconstruction with these tendons. Further modulation of this regeneration may further reduce graft harvesting morbidity.

Keywords: hamstring; regeneration; anterior cruciate ligament (ACL)
hamstring tendons from a proximal to distal fashion. By 15 months, MRI characteristics on T1- and T2-weighted images to the level of the distal 1 cm to 2 cm of the native tendon were normal. The senior author has previously shown that the histology and physiology of regenerated semitendinosus tendons are similar to native tendons with an ultimate strength of 23% and 62% of normal tendons at 16 and 28 weeks, respectively. A distinct tendon at the distal musculotendinous junction, with an apparent attachment distally on the tibia, was noted at 16 weeks. Preliminary histologic evaluation at 16 weeks demonstrated fatty atrophy of the muscle and longitudinal, wavy-appearing yet well-organized collagen at 28 weeks. These previous 2 studies, however, evaluated the regenerate tendon only and not the entire regenerate muscle-tendon unit.

In this third phase of the study, we evaluated the long-term properties of the proximal musculotendinous junction, the regenerate tendon, and the distal tendinous insertion into bone in the same rabbit model. Prior studies have only accounted for short-term regeneration without the remodeling that takes place with time and stress across the muscle-tendon-bone unit. Furthermore, sufficient time for regeneration is necessary to characterize the neo-tendon as scar with tendon-like properties versus a product that physiologically acts as a true tendon. Hamstring regeneration is advantageous because the regenerated tissue may serve as a graft to surgically reconstruct the knee and restore stability without creating problems that may arise from autograft tissue. Harvesting of autografts can cause alterations in knee joint motion and kinematics of the lower extremity, which can drastically compromise the outcome of a surgical procedure. If the autograft regenerates and has a similar structure and function to the native tendon, however, it may prove to be the ideal choice for reconstructive procedures.

**MATERIALS AND METHOD**

Thirty-five New Zealand white rabbits weighing an average of 4.5 kg to 5.0 kg were used for the semitendinosus tendon harvest. Approval for all experiments was obtained by the institutional Animal Care and Use Committee. After induction of anesthesia with intramuscular injections of ketamine (40 mg/kg) and xylazine (3 mg/kg), the skin overlying the knees was cleansed and shaved with 70% alcohol solution. The animals were kept anesthetized throughout the testing period with intermittent intramuscular doses of ketamine. Intramuscular cefazolin (15 mg/kg) was used for antimicrobial prophylaxis. Right and left knees were alternated to avoid the role dominance may play as a confounding variable. A medial parapatellar incision was made, the distal semitendinosus tendon was dissected free, and a whipstitch was placed in the distal aspect of the tendon just proximal to its insertion before the tendon was sharply released from its insertion at the proximal tibia. Using a tendon-stripping device, the tendon was removed in a manner identical to that for ACL reconstruction. The wounds were closed with absorbable suture, and the rabbits were allowed full caged activity without immobilization. The objectives were to examine the regenerated hamstring as follows: (1) histologically, to determine tissue organization, particularly that of the cells and surrounding collagen fibrils; (2) biochemically, to establish tissue composition in relation to the normal hamstring; (3) physiologically, to determine the function of the musculotendinous unit assayed by electrical stimulation of the regenerate tissue; and (4) biomechanically, by loading to failure. During a period of 9 to 12 months postoperatively, semitendinosus muscle bellies and the regenerate tendons, with their distal bony attachments, were retrieved for evaluation of the regenerate muscle-tendon-bone unit. The musculotendinous unit harvested from the contralateral knee served as the control tendon.

**Histologic Analysis**

Histologic character and organization of the regenerate were evaluated with hematoxylin and eosin staining and then analyzed with a standard light microscope (native = 4, regenerate = 8). The bone-tendon interface was also stained with safranin O. In addition, type I collagen was immunolocalized in the native and regenerate tendon (3 rabbit pairs). Freshly harvested tissue was preserved in Optimal Cutting Temperature Compound (Sakura, Torrance, Calif) and 10-µm sections were prepared using a Leica CM3050 cryomicrotome (Leica, Bannockburn, Ill) at −30°C. Sections were incubated with monoclonal mouse anti-type I collagen (Sigma, St Louis, Mo), followed by fluorescein isothiocyanate labeled goat antimouse IgG (American Qualex, San Clemente, Calif). Fluorescence was visualized with a Nikon Eclipse E600 microscope (Nikon, Melville, NY) equipped with a digital camera and Adobe Photoshop 5.0 software (Adobe Systems Inc, San Jose, Calif). Vascularity of the regenerate tendon was then assayed with platelet/endothelial cell adhesion molecule (PECAM-1) antibody (Santa Cruz Biotechnology, Santa Cruz, Calif), an anti-CD31 antibody, because CD31 is a membrane polypeptide found in blood vessel walls (3 rabbit pairs). Tissue was paraffin embedded and 10-µm sections were obtained using a Leica RM2125 microtome. Sections were rehydrated and endogenous peroxidase activity was destroyed with methanol and 30% hydrogen peroxide. To reveal antigens in the paraffin-embedded specimens, slides were placed in an antigen unmasking solution (Vector, Burlingame, Calif). Slides were incubated with an avidin blocking solution, and the primary antibody, goat anti-PECAM-1, was then added to each slide. A horse antigoat secondary antibody was applied, followed by the avidin biotinylated enzyme complex (Vector) and the 3-3′ diaminobenzidine substrate (DAKO Corp, Carpinteria, Calif). After the diaminobenzidine incubation, the slides were counterstained in hematoxylin and then dehydrated. Again, staining was visualized with a Nikon Eclipse E600 microscope equipped with a digital camera and Adobe Photoshop software.

**Ultrastructural Analysis**

Regenerate and native hamstring tendons of 2 rabbits were dissected from the animal, cut into 1-mm thick
pieces, and placed overnight in 0.1-M phosphate buffer with 2.5% glutaraldehyde and 4% paraformaldehyde. The tissue was then fixed in 1% osmium tetroxide, dehydrated in graded acetones, and flat-embedded in Epon plastic 812 (Ernest F Fullam, Inc, Latham, NY) in a cross-sectional orientation. The 85-nm sections were obtained and stained with 0.25% lead citrate and 5% uranyl acetate in 50% acetone and then observed and photographed in a JEOL 100CX transmission electron microscope (CEMM Instruments, Los Angeles, Calif). All pictures of cross-sectional tendons to be measured were taken at ×26 000 magnification and printed with an enlargement factor of 3.

Biochemical Composition

Amino sugars and hydroxyproline (Hypro), as indicators of glycosaminoglycan (GAG) and collagen, respectively, were measured in 5 normal and 4 regenerate tendons excised after biomechanical testing. After extraneous soft tissue was removed, tendons were diced and placed in sterile papain (Sigma) buffer solution (125 μg/ml in 1X PBE, pH 6.5) at 60°C for 24 h. The DNA was analyzed using the Hoechst 33258 dye (Sigma) assay with a calf thymus DNA standard. For Hypro, amino sugars, as a measure of GAG, were determined using the dimethylmethylene blue (Cresent Chemicals Corp, Hauppauge, NY) colorimetric assay with dermatan sulfate (Sigma) as a standard. For Hypro, an aliquot of the papain digest was hydrolyzed in 6 N HCl for 16 h at 110°C using a single-factor analysis of variance with tendon type (native vs regenerate) serving as the independent variable and a statistical significance level of P < .05.

Biomechanical Testing

The same tendons used for electrical stimulation were then tested in static tensile tests to failure. After the tendon and limb were removed from the machine, the proximal origin (ischial attachment) of the semitendinosus was detached from the hindlimb. Again, all tissue was kept moist with phosphate-buffered saline throughout the procedures. The free tendinous unit was remounted in the Instron unit, ensuring that the tendon was oriented perpendicular to the direction of testing. The tendon was preloaded to 0.01 N and the gauge length (length of specimen between the grips) was recorded using precision dial calipers (Mitutoyo Model 505-646, Mitutoyo Corp, Japan). Tensile testing was then conducted to failure at a constant strain rate of 15% per second. Load versus extension data were sampled and recorded at a frequency of 10 Hz and with every change in load of 0.05 N. Maximum load to failure, stiffness (slope of the linear region of the load vs extension curve), and energy absorbed to failure (area under the curve) were compared between groups using a single-factor analysis of variance with tendon type (native vs regenerate) serving as the independent variable and a statistical significance level of P < .05. The area under the curve was measured by the American Innovation Videometric 100 computer (American Innovation, Inc, San Diego, Calif).

RESULTS

Of the 35 rabbits, 31 rabbits were available for testing. One rabbit expired during the initial surgery because of complications from anesthesia (respiratory depression). Three additional rabbits expired before the 9-month minimum follow-up time. Two rabbits had a wound dehiscence with a superficial infection treated with intramuscular cefazolin. The regenerate tendon formed in 26 of the remaining 31 rabbits (84%). This regenerate was completely indi-
tinct in 5 specimens, 2 of which had suffered the periopera-
tive infection and wound dehiscence. By gross examina-
tion, the neo-tendon was found to be highly variable in its
size and location of insertion (Figure 1). When the contra-
lateral tendon was larger, a more significant regenerate
tendon developed. Most regenerate tendons were more
superficial in insertion than the native semitendinosus
tendon. The tendons were widely distributed with respect
to insertion site and proximity to the joint line. The mus-
culotendinous junction, with gross examination, also
revealed fatty degeneration similar to the fatty atrophy of
the semitendinosus muscle belly on the ipsilateral side.

Under microscopic analysis, fibril arrangement was
noted under hematoxylin and eosin staining, and type I
collagen was immunolocalized to the tendon fibrils of both
native and regenerate tissue (Figure 2). There was, howev-
er, a considerable difference between the staining patterns
of the regenerate compared with the native. The discrete
orientation in the regenerate was visualized, although to a
lesser magnitude than the native tendon. In other areas,
the pattern of longitudinal orientation was not as apparent
in the regenerate tendon but demonstrated areas of
increased cellularity instead. Further biologic heterogene-
ity of the neo-tendon could be visualized by the differences
in alignment, cellularity, and composition due to the vari-
able rate of regeneration in this animal model (Figure 3).
In addition, there was a marked difference between native
and regenerate tendon in the relative hypervascularity of

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**Figure 1.** Gross morphology of regenerate (R) and native (N) rabbit hamstring tendon. Compared with the native tendon, the regenerate tendon is highly variable in size and was found to attach to the tibia at variable locations.

**Figure 2.** Histology of native tendon and regenerate tendon and immunolocalization of type I collagen identifying the differences in tissue organization and cellularity. A, native tendon; B, regenerate tendon; i, Cryosection of tendon stained with hematoxylin and eosin (x40 magnification); ii, Tendon stained with hematoxylin and eosin (x200 magnification); iii, Tendon treated with anti–type I collagen antibody.
the neo-tendon as witnessed by anti-PECAM-1 staining. The mean number of endothelial cells in the native was 5 (SD = 0), whereas the mean number in the regenerate was 78 (SD = 18.93). Furthermore, the orientation of the cells at the junction of the tendon and bone was clearly visible (Figure 4). The cells in the tendon-bone interface were aligned along the direction of fibers in the native tendon (Figure 4A), whereas the cells were less well organized at the tendon-bone interface of the regenerate tendon (Figure 4B). Ultrastructural analysis with transmission electron microscopy showed the collagen fibrils to be well organized, but overall, they were smaller in diameter with less variance. The native tendon measured 126 nm ± 39 nm, and the regenerate was 42 nm ± 32 nm. This difference in diameter was statistically significant (P < .001). The surface topography under electron microscopy revealed homogeneous, rounded fibrils (Figure 5). With Safranin O staining, matrix GAGs were identified at the bone-tendon interface (Figure 4). The biochemical assay confirmed the production of Hypro and proteoglycan in the extracellular matrix but at significantly lower levels in the regenerate tendon (Table 1).

The physiological properties of the regenerate tendon, as determined by electrophysiological and biomechanical testing, revealed significant differences between the native tendon and the neo-tendon. When the muscle-tendon complex was stimulated to tetany, causing all forces to be loaded at the musculotendinous junction, the mean stimulation load for the native tendon was 5.41 N (SD = 2.24), whereas the regenerate only sustained 1.35 N (SD = 0.91). Thus, the regenerate muscle and musculotendinous junction was capable of creating and sustaining an average of 25% of the maximum load when compared with the native side (P = .002). Biomechanical parameters indicated that
the ultimate tensile strength, the energy sustained before rupture, and the stiffness of the regenerate were all significantly different from the native tendon (Table 2 and Figure 6).

**DISCUSSION**

Hamstring grafts have been increasingly used for ACL reconstruction,6 and the need for additional autologous graft tissue is increasing with the rise of revision ACL and retear of the ACL graft. The hamstring graft has traditionally been useful in treating patients with patellofemoral arthrosis, patellofemoral arthralgia, and a history of anterior knee pain, as well as in revision surgery following patellar tendon harvest.5,11,18 Concerns regarding decreased hamstring strength postoperatively, however, bring into question the utility of hamstring graft. Proponents of hamstring tendon grafts state that decreased donor site morbidity and accelerated rehabilitation favor use of the hamstring graft; recent literature also supports the biomechanical advantage of quadrupled hamstring tendon grafts when compared with bone–patellar tendon–bone, another common ACL graft.19 In addition, the patellar tendon graft has had reports of chondrosis,1,37,38 patellar fracture,9,31 patellar tendon rupture,3,30 and even combined fracture and tendon rupture.4,33 Persistent donor-site morbidity, such as tenderness, anterior knee pain, and difficulty kneeling, exists in 40% to 60% of patients who have undergone ACL reconstruction with patellar tendon autografts.31

The existence of an autograft that regenerates and leaves minimal dysfunction is extremely appealing. Despite some authors who have found a loss of hamstring strength after harvest for ACL reconstruction,24,29 most authors have established no significant difference between the operated and nonoperated side.27,40,41 A compensatory hypertrophy of the nonharvested knee flexors did not exist,40 so the maintenance of knee flexor strength may be

<p>| TABLE 1 |
|-----------------|-----------------|-----------------|-----------------|
| <strong>Biochemical Analysis of Native and Regenerate Hamstring Tendon</strong> |</p>
<table>
<thead>
<tr>
<th><strong>Ratio (µg/µg)</strong></th>
<th>Native (n = 5)</th>
<th>Regenerate (n = 4)</th>
<th><strong>P Value</strong></th>
</tr>
</thead>
<tbody>
<tr>
<td>GAG/DNA</td>
<td>2219 ± 1082</td>
<td>828 ± 266</td>
<td>.02</td>
</tr>
<tr>
<td>Hypro/DNA</td>
<td>9038 ± 1956</td>
<td>2196 ± 112</td>
<td>.001</td>
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*a Amino sugars and hydroxyproline (Hypro), as indicators of both glycosaminoglycans (GAGs) and collagen, were determined in relation to the total DNA content per cell.

*b Statistically significant.

<p>| TABLE 2 |
|-----------------|-----------------|-----------------|-----------------|
| <strong>Biomechanical Properties of Native and Regenerate Tendon</strong> |</p>
<table>
<thead>
<tr>
<th><strong>Maximum load, N</strong></th>
<th>Native (n = 6)</th>
<th>Regenerate (n = 6)</th>
<th><strong>P Value</strong></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>33.69 ± 9.78</td>
<td>10.64 ± 10.31</td>
<td>.002</td>
</tr>
<tr>
<td><strong>Energy to failure, N</strong></td>
<td>0.44 ± 0.10</td>
<td>0.28 ± 0.11</td>
<td>.023</td>
</tr>
<tr>
<td><strong>Stiffness, N/mm</strong></td>
<td>11.90 ± 6.25</td>
<td>3.73 ± 4.97</td>
<td>.031</td>
</tr>
</tbody>
</table>

*a Statistically significant.
attributed to the effective regeneration of the harvested semitendinosus and gracilis tendons. Ample documentation of regeneration of the harvested hamstring tendons exists in the literature. Papandrea et al. used ultrasound to document the uniform echostructure within the regenerate tendon, which was similar to the native tendon at 18 to 24 months after harvest. And MRI documentation also exists in the scientific literature. Histologic confirmation of a tendinous tissue was provided by Eriksson et al. and Ferretti et al. Often, the regenerate was found to have variable insertion with respect to the joint line, inserting more proximal, medial, and superficial than the contralateral native hamstring tendon. This same finding of a variable distal insertion point was also noted in our study. A significant loss of internal rotation force after hamstring harvest, however, is attributable to this variant regenerate insertion site. Thus, the protective mechanism that the hamstrings play in decreasing forced external rotation is lost.

In a human study by Eriksson et al., 75% of patients showed regeneration of their semitendinosus tendons. In our study, 84% of rabbits had regeneration of their semitendinosus tendons. Complete maturation of the regenerate tissue can only be achieved when it is subjected to adequate mechanical stresses. Thus, the importance of allowing the animals unrestricted caged activity is emphasized. Under gross examination, the regenerate was quite variable in size, which was manifest in the biomechanical testing. The regenerate that formed was found to be inferior or with respect to ultimate tensile load and energy absorbed before failure. Of the 6 regenerate tendons on which tensile strength measurements were obtained, the range of values was from 20% to 75%, with an average value of 32% of the native's strength. This difference may have been attributable to the immaturity of the specimen even though the regenerate was given 9 to 12 months to regenerate and remodel. Of note, a native contralateral tendon with an ultimate tensile load of less than 22 N usually resulted in a weak, wispy regenerate. A native contralateral tendon with an ultimate tensile load of greater than 35 N, however, resulted in a regenerate at least 25% of the original strength, and sometimes up to 75%. Thus, a threshold size of tendon must exist before any significant regeneration can occur. One limitation of these measurements is that ultimate load and energy absorbed are structural properties that reflect and depend on both tendon geometry and tendon material properties. As there was no precise way to measure the cross-sectional area of these small, irregular tendons, load data could not be converted to stress data, and pure material properties (maximum stress to failure, modulus of elasticity, strain energy density) were not analyzed. However, inferior material behavior, correlating to stiffness, was identified in the regenerate tendon produced. The difference in stiffness may have been a result of diminished or incomplete collagen cross-linking, another factor implicated in nascent tendons. The immaturity of the tendon could also be noted by the disorderly arrangement of the tendon insertion site into bone, the hypervascular tissue found in the neo-tendon (similar to the initial phases of wound healing), and the variability with which the tendon regrows (Figure 3). Ample type I collagen was found in the regenerate, as visualized by the immunohistochemical staining (Figure 2B), but once again, the organization was not as symmetric when compared with the native. Type III collagen has the ability to rapidly form cross-linked intermolecular disulfide bridges, which may have assisted in the initial stability of the regenerate. These observations, in addition to the finding that collagen fibril diameter is thinner overall in the regenerate tendon, are consistent with the weaker mechanical properties of the regenerate compared to the native tendon.

Further evidence that the regenerate is not just fibrous scar but a functioning tendon is demonstrated by the electrophysiological testing. In our model, by stimulating the muscle to tetany, the force was concentrated at the musculotendinous junction. Because force could be transmitted through the muscle-tendon complex, reestablishment of the connection between the tendon and muscle, such as via the basement membrane, is likely. Otherwise, when the load was concentrated at the musculotendinous junction, rupture in the muscle-tendon region would occur, and the distal tendon would not receive any significant load.

Biochemical markers can also assay the nature of the regenerate. Oxidative potential estimated by citrate synthase activity has been used. These authors, however, used the muscle and not the regenerate tendon for their testing. Kartus et al. used GAG content to evaluate biopsy specimens from the patellar tendon 27 months after the harvesting procedure. No appreciable amount of GAG was found in the biopsy specimen. The bone-tendon interface in our study, however, showed evidence of proteoglycan in the less ordered matrix of the regenerate when compared with the organized matrix in the native tendon. In addition, with the biochemical assay, Hypro and GAG (amino sugars) were identified in the regenerate tendon, corresponding to collagen and proteoglycan, respectively. The content of these extracellular matrix elements was, however, significantly lower. Even though these extracellular matrix proteins were indeed synthesized in the neo-tendon, their lower levels of production or increased degradation may have been a factor in the subsequently weaker regenerate.

We were able to demonstrate a functional regenerate tissue that histologically appears to be tendon rather than just organized scar. The regenerate was found to react physiologically similar to the native tendon. The question of how the tendon regenerates without a discrete remaining paratenon still remains. The purported theory is that the regeneration occurs along "fascial planes." The normal gracilis and semitendinosus tendons lie in the fascia of the medial aspect of the knee. This fascia forms a 3- to 4-cm band around the tendons 8 cm to 10 cm proximal to the pes anserinus. This area has been shown to connect with the medial intermuscular septum and the sheath investing the semitendinosus. This tissue plane for the semitendinosus may help explain the proximal to distal regeneration of the semitendinosus tendon. Furthermore, the postharvest
hematoma in this sepal plane may act as a scaffold for fibroblast precursor cells to start collagen production.6

Further study is needed to characterize the neural innervation to the regenerate tendon, especially at the musculotendinous junction, and to evaluate the properties of the basement membrane at the muscle-tendon interface. An even longer follow-up can be used to allow further maturation of the regenerate tendon before beginning testing and research. In addition, vascular identification of the regenerate can help define the source of blood vessel input into the neo-tendon. Preliminary analysis with PECAM-1 demonstrated abundant vascularity when compared with the native tendon. In this animal model, a majority but not all hamstrings regrew, the regrowth was affected by original hamstring size and wound complications, and the regrowth had variable size and insertion points that may not be able to reproduce the full function of the hamstrings in vivo. We speculate that treatment with growth factors could enable a viable, robust graft to be consistently produced that may allow further restoration of function. However, further research on humans will be needed to elucidate the function of the regenerated in vivo.

ACKNOWLEDGMENT

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REFERENCES


