Sustained Delivery of Transforming Growth Factor Beta Three Enhances Tendon-to-Bone Healing in a Rat Model

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ABSTRACT: Despite advances in surgical technique, rotator cuff repairs are plagued by a high rate of failure. This failure rate is in part due to poor tendon-to-bone healing; rather than regeneration of a fibrocartilaginous attachment, the repair is filled with disorganized fibrovascular (scar) tissue. Transforming growth factor beta 3 (TGF-β3) has been implicated in fetal development and scarless fetal healing and, thus, exogenous addition of TGF-β3 may enhance tendon-to-bone healing. We hypothesized that: TGF-β3 could be released in a controlled manner using a heparin/fibrin-based delivery system (HBDS); and delivery of TGF-β3 at the healing tendon-to-bone insertion would lead to improvements in biomechanical properties compared to untreated controls. After demonstrating that the release kinetics of TGF-β3 could be controlled using a HBDS in vitro, matrices were incorporated at the repaired supraspinatus tendon-to-bone insertions of rats. Animals were sacrificed at 14–56 days. Repaired insertions were assessed using histology (for inflammation, vascularity, and cell proliferation) and biomechanics (for structural and mechanical properties). TGF-β3 treatment in vivo accelerated the healing process, with increases in inflammation, cellularity, vascularity, and cell proliferation at the early timepoints. Moreover, sustained delivery of TGF-β3 to the healing tendon-to-bone insertion led to significant improvements in structural properties at 28 days and in material properties at 56 days compared to controls. We concluded that TGF-β3 delivered at a sustained rate using a HBDS enhanced tendon-to-bone healing in a rat model. © 2011 Orthopaedic Research Society. Published by Wiley Periodicals, Inc. J Orthop Res 29:1099–1105, 2011

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Surgically repaired rotator cuff tears are prone to recurrent tears, with a failure rate reaching 94% for large tears.[1,2] This high rate of failure is due to the fact that the healing process of tendon to bone is reparative (i.e., scar-mediated) and not regenerative. The composition and structural organization of the repaired tissue does not mimic the original uninjured tissue. Whereas most uninjured insertions have a fibrocartilage transition between tendon and bone,[3,4] repaired tendons lack a transition zone. Instead, the interface is filled with disorganized fibrovascular connective tissue whose mechanical properties are inferior to that of the uninjured tissue.[5–8] Although the large quantity of this fibrovascular tissue leads to a significant increase in the cross-sectional area relative to that of the normal insertion, the mechanical properties (normalized to cross-sectional area and thus indicative of the "quality" of the tissue) of the repaired tendon are inferior.[5]

Fetal wounds in skin heal in a regenerative manner (the repaired tissue is identical to the original tissue), while adult wounds heal in a reparative manner (i.e., via a scar-mediated process).[9–11] Several studies compared healing properties of adult to fetal tissues and identified a pattern of growth factor expression unique to development.[9,10,12] For transforming growth factor β (TGF-β) isoforms, fetal wound healing is characterized by low expression of TGF-β1 and TGF-β2, high expression of TGF-β3, and no scar tissue. Conversely, adult wound healing is characterized by high levels of TGF-β1 and TGF-β2, low levels of TGF-β3, and extensive scar.[9–12] Injections of exogenous TGF-β3 to the site of a nascent dermal wound reduce scar formation, thus implicating TGF-β3 in scar-less fetal and adult wound healing.[9–11] Similar temporal expression patterns are seen at developing and healing tendon-to-bone insertions.[8,13]

Based on these studies, we hypothesized that sustained delivery of TGF-β3 would promote regenerative healing over reparative healing at the adult tendon-to-bone insertion site. This would be evident as reduced fibrovascular scar tissue formation, improved structural organization, and improved mechanical properties in TGF-β3-treated repairs compared to controls that received surgical repair alone. To test this hypothesis, we delivered TGF-β3 using a heparin-binding delivery system to the supraspinatus tendon-to-bone repair sites in rat shoulders and evaluated repairs using histological and biomechanical outcomes (Fig. 1).

METHODS

Growth Factor Delivery System

Bolus application of growth factors typically leads to clearance from the wound site within 48 h.[10] Given that fibroblast cells begin to infiltrate the wound after 2–4 days, acute TGF-β3 delivery would have limited effectiveness. We previously used fibrin matrices containing a heparin-based delivery system (HBDS) to control the release of heparin-binding growth factors.[14,15] Release occurs through "passive" dissociation from the delivery system and diffusion from the matrix, as well as through "active" cell-mediated degradation of the fibrin matrix. Both mechanisms were evaluated in vitro.

TGF-β3 Passive Release Kinetics—Acellular Study

To determine the passive (acellular) release kinetics of TGF-β3 from the delivery system, 400 μl fibrin matrices were made in quadruplicate ($N = 4$) in 24-well tissue culture plates as described previously.[14] The matrices consisted of 50 ng TGF-β3 (R&D Systems, Minneapolis, MN), 20 mg/ml fibrinogen

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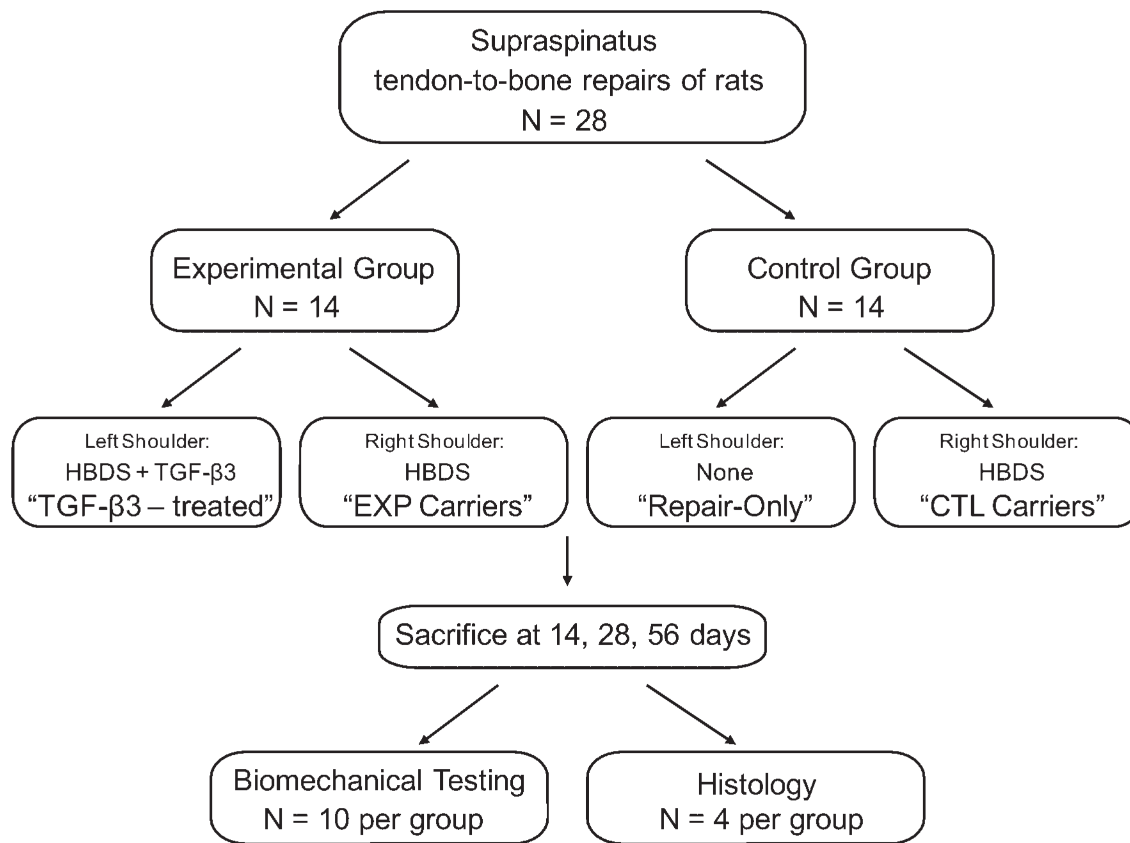

Figure 1. Study design. Left shoulders received matrices loaded with 100 ng of TGF-β3; right shoulders received matrices lacking growth factor. A second group of control rats was used to examine potential systemic effects of the growth factor and for comparison to surgical repair only. Animals were euthanized at 14 days for histology and at 28 and 56 days for histology and biomechanical testing.

(EMD Biosciences, San Diego, CA), 25 mg/ml peptide (0.16 mM—dLNQEQVSPK[βA]FAKLAARLYRKA-NH_2, where dL denotes dansyl leucine), 45 mg/ml heparin, 50 mM $CaCl_2$, 12.5 U/ml thrombin, and Tris-buffered saline (TBS; 33 mM Tris, 137 mM NaCl, 2.7 mM KCl, pH 7.4). The heparin concentration in the fibrin matrices varied from 0 to 4 mM to yield the desired growth factor to heparin molar ratios (1:0, 1:100, 1:1,000, and 1:10,000). The heparin/peptide ratio remained constant at 1:10. To examine growth factor release into the media, a series of washes with TBS were performed over 10 days, as described previously.[14] An ELISA for TGF-β3 (R&D Systems) was then performed on all wash volumes and on the remaining fibrin. To compare the passive release kinetics of the matrices with and without the delivery system, the ratio of TGF-β3 released into the buffer per day from the HBDS group to the amount released from the no HDBS group was calculated.

TGF-β3 Active Release Kinetics—Cellular Study

To examine cell-mediated release, fibroblasts were isolated from the supraspinatus and infraspinatus tendons of Sprague–Dawley rats ($N = 8$ cell isolations) as described previously.[14] Fibrin matrices were then seeded with the fibroblasts, and the active TGF-β3 release rate was determined. The procedure was identical to that of the acellular study methods with the following exceptions: gels were seeded with 50,000 cells in culture medium (1% FBS DMEM) after the first day of washes; daily washes were performed using 1% FBS DMEM to maintain cell viability; and experimental groups included matrices containing the delivery system at a factor/heparin molar ratio of 1:1,000 (delivery system group) and matrices lacking the delivery system (ratio of 1:0) (no delivery system group). Matrices were made in triplicate for all groups. The experiment was repeated for eight cell isolations. To compare the passive release kinetics of the matrices with and without the delivery system, the ratio of TGF-β3 released into the buffer per day from the HBDS group to the amount released from the no HDBS group was calculated.

Statistical Analysis

A one-way ANOVA with planned polynomial contrasts was used to compare the TGF-β3 ratios over time. Paired t-tests were used to compare the release of TGF-β3 from gels with and without the HBDS at each timepoint. $p < 0.05$ was considered significant.

Animal Model

All animal procedures were approved by the institutional Animal Studies Committee. Supraspinatus tendons in bilateral shoulders of 76 adult male Sprague–Dawley rats (375–400 g) were transected and repaired to the humeral head using 5–0 proline suture, as described previously.[5,8] In one group, fibrin matrices with the HBDS (+/− TGF-β3) (30 μl matrix volume) were incorporated bilaterally at the supraspinatus tendon-to-bone repair sites by passing the suture through the matrix before completing the repair. The left shoulders received matrices that were loaded with 100 ng of TGF-β3 (100 μg/ml, 1:1,000 growth factor to heparin molar ratio), while the right shoulders received matrices lacking the growth factor and

served as contralateral (carrier) controls (Fig. 1). A second group was used to control for potential systemic effects of the growth factor and to serve as a comparison group to conventional treatment (i.e., surgical repair only). The left shoulders of these rats were repaired surgically but did not receive any matrices. The right shoulders received matrices lacking the growth factor (i.e., carrier controls). Animals were euthanized 28 and 56 days postoperatively ($N = 11$–16 per group) for biomechanical testing. Animals were euthanized at 14, 28, and 56 days postoperatively for histology ($N = 4$ per group) (Fig. 1). A separate group of uninjured age-matched rats ($n = 8$) was used as normal controls.

Histology-Based Assays

Four specimens from each group were examined. Specimens were fixed, decalcified, embedded in paraffin, and sectioned at 5 μm in the coronal plane. Sections were stained with toluidine blue to examine fibrocartilage, hematoxylin and eosin to examine cell morphology, and Masson's trichrome to examine fibrous tissue. Proliferating cell nuclear antigen (PCNA) immunohistochemistry was done in accordance with the manufacturer's protocol (Zymed Labs, San Francisco, CA) as a measure of cell proliferation. The antibody was visualized by incubation with diaminobenzidine (DAB). Three independent observers (NH—pathologist, LG—orthopedic surgeon, and CM—graduate student), blinded to group and timepoint, evaluated tissue sections for cellularity, cell proliferation, vascularity, inflammation, fibrosis (scar tissue formation), and fibrocartilage formation.[5,8] A standard scoring system was used to determine the levels of each outcome (− no prevalence, + minimal prevalence, ++ mild prevalence, +++ moderate prevalence, ++++ high prevalence). Our analysis was focused solely on the healing tendon-to-bone interface.

Geometry and Biomechanics

Biomechanical testing of the supraspinatus tendon-to-bone insertions was performed as previously described.[5] The thickness of each insertion was measured using a laser displacement sensor (Keyence LK-081, Wooddiff Lake, NJ), and the width was measured using optical methods. The cross-sectional area was calculated by assuming an elliptical cross-section. To determine the mechanical properties of the healing insertion without the confounding effect of the suture repair strength, sutures were cut at the entrance of humeral head bone tunnel prior to testing. To control hydration, temperature, and pH during testing, tendons were immersed in a physiologic saline bath at 39°C (rat body temperature) during testing. A servo-hydraulic materials testing system (Instron Corp., Model 8841 Norwood, MA) was used to perform the test (i.e., preconditioning, followed by stress relaxation, followed by constant ramp at a strain rate of 0.1%/s until failure). Engineering stress was calculated by dividing the tensile force by the initial cross-sectional area of the tendon. Strain was measured optically by tracking two surface stain lines using custom-written software (Matlab, Natick, MA).[5] Ultimate load, stiffness (the slope of the linear portion of the load-deformation curve), toughness (the area under the load-deformation curve), ultimate stress, and tangent modulus (the slope of the linear portion of the stress–strain curve) were determined.

Statistical Analysis

For geometric and biomechanical outcomes, groups were compared using a two-factor ANOVA for time and treatment followed by a Fisher least-squares differences post hoc test. Paired data (TGF-β3 group vs. experimental group carriers or repair-only group vs. control group carriers) were compared using paired t-tests. $p < 0.05$ was considered significant.

RESULTS

In Vitro Studies

Sustained growth factor release was achieved in the absence of cells via the HBDS (Fig. 2A). The % change of TGF-β3 released from the gels containing the HBDS to those lacking the delivery system was initially negative, indicating that more growth factor was being released from the no-HBDS gels at the early timepoints. Over time; however, the % change was positive, indicating that the HBDS gels were releasing more growth factor. The amount of TGF-β3 released from the two groups were significantly different at days 0, 1, 2, 5, 7, and 9. Similarly, the amount of growth factor remaining in the HBDS gels at the end of 10 days was significantly more than the amount remaining in the no-HBDS gels. Moreover, the ratios of TGF-β3 released from the gels with and without the HBDS significantly increased over time. Gels without the HBDS (1:0) released nearly all (99%) of the growth factor by day 10, whereas the gels with the HBDS at ratios of 1:100, 1:1,000, and 1:10,000 released 88%, 74%, and 52%, respectively. The same trends were seen in tendon fibroblast seeded gels (Fig. 2B), although the release rate was accelerated in

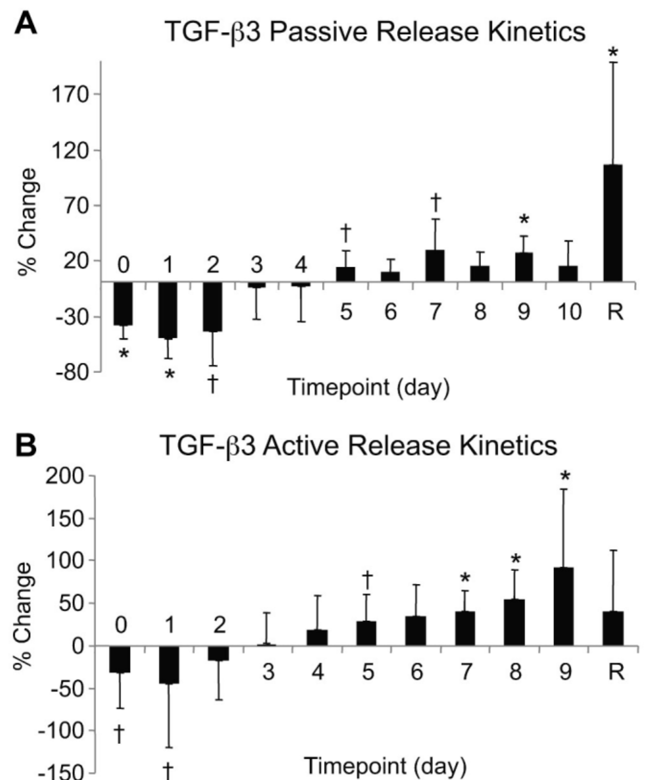

Figure 2. In vitro release kinetics. In both the acellular (A) and cellular (B) environments, the ratios significantly increased with time, indicating sustained release of TGF-β3 from the gels containing the delivery system. Differences in the amount of TGF-β3 released from the two groups (i.e., HBDS vs. no-HBDS) are signified by stars and crosses ($^*p < 0.05$, $^†p < 0.1$). R: TGF-β3 remaining in the gels at the end of the 10-day experiment.

Table 1. Histological Assessment

Group	Fibrosis	Inflammation	Cellularity	Proliferation	Vascularity	Fibrocartilage
14 day						
TGF-β3	+++	+++	+++/++++	+++	+++	−
Exp carrier	+	++	++/+++	+/++	++	−
Ctl carrier	++	+	++/+++	++	++	−
Repair only	++	++	++	++/+++	++	−
28 day						
TGF-β3	++	+	++	−/+	++	−
Exp carrier	++	−/+	++	−/+	+	−
Ctl carrier	+	−/+	+/++	+	+	−
Repair only	+	+	++	+/++	+/++	−
56 day						
TGF-β3	++	−/+	++	−/+	++	−
Exp carrier	+/++	−/+	+	−/+	+	−
Ctl carrier	+	−/+	+	−/+	+	−
Repair only	+/++	−/+	+	−/+	+	−

the presence of cells. The amount of TGF-β3 released from the no-HBDS group was greater than the amount released from the HBDS group at the early timepoints. At the later timepoints; however, the opposite trend was observed. TGF-β3 release from the HBDS was significantly increased compared to the no-HBDS group at days 7, 8, and 9. A significant linear increase in these ratios (HBDS/no-HBDS) was observed over time.

In Vivo Studies—Histology

Good agreement was found among the results from the blinded investigators. Large discrepancies in grades were resolved by re-evaluating the slide as a group. Blind evaluation revealed increased cellularity and increased cell proliferation at the repair site in the TGF-β3 group at 14 days (Table 1 and Fig. 3). The number of blood vessels (vascularity) and the amount of fibrous scar tissue was also substantially increased in the TGF-β3 group at the earliest timepoint (day 14). An increased immune response (an increase in lymphocytes) was apparent in the TGF-β3-treated tendons compared to all other groups at 14 days. The amount of scar tissue and the number of cells and blood vessels remained high at all timepoints in the TGF-β3-treated tendons, whereas differences in lymphocytes and cell proliferation were no longer evident at 28 and 56 days. No evidence of fibrocartilage regeneration was seen in any of the groups at any timepoint (Table 1).

In Vivo Studies—Geometry

At 28 days, the cross-sectional areas of the tendons treated with TGF-β3 were significantly greater than that of any other group (Fig. 4). At 56 days; however, the TGF-β3-treated tendons had the smallest cross-sectional area. The areas in the control group (i.e., control group carriers and repair-only group) increased significantly over time, whereas those in the experimental group (i.e., TGF-β3 treated and experimental group carriers) did not. Cross-sectional areas of all repaired tendons were significantly increased compared to normal/uninjured tendons at both timepoints.

In Vivo Studies—Biomechanics

At 28 days, ultimate load of the TGF-β3-treated tendons was significantly increased compared to the contralateral control tendons ($p = 0.04$, 30% increase, Fig. 5A). There was a trend towards increased ultimate load in the TGF-β3-treated group compared to the

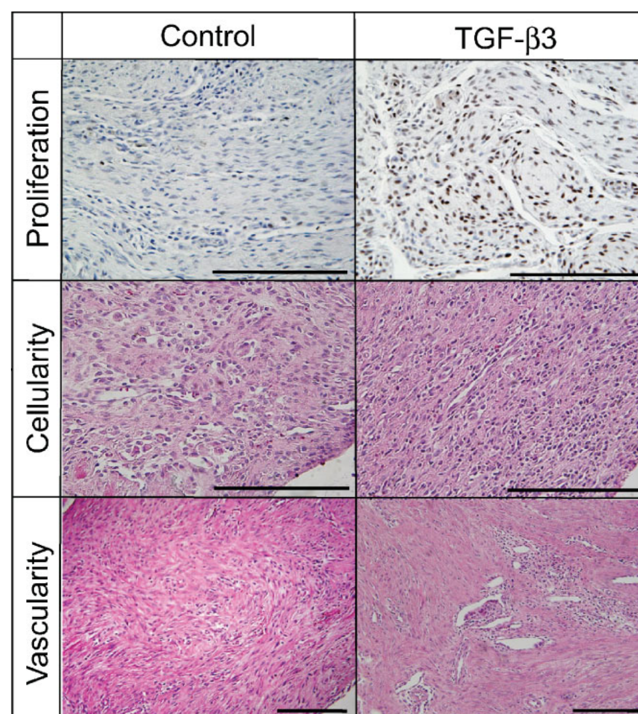

Figure 3. Histology. Sections stained with PCNA (top panels; cells in the proliferative stage of the cell cycle stain dark brown) and hemotoxylin and eosin (middle and bottom panels) at 14 days. The tendons treated with TGF-β3 exhibited increases in cell proliferation, cellularity, and vascularity compared to controls (i.e., experimental group carriers) [scale bars = 200 μm]. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com]

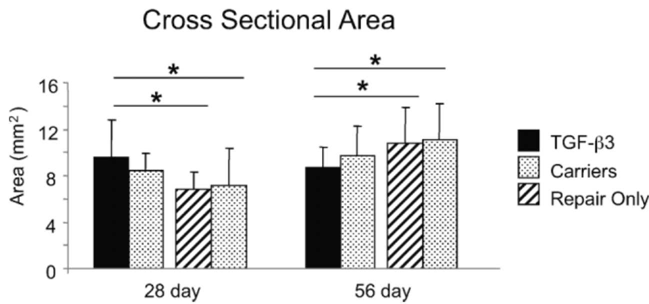

Figure 4. Geometry. Cross-sectional area of TGF-β3-treated tendons was increased at 28 days compared to control group carriers and repair-only tendons. At 56 days, the area of TGF-β3-treated tendons was decreased compared to control group carriers and repair-only tendons. The areas of repaired insertions were increased compared to normal/uninjured insertions (normal = 2.6 ± 0.4 mm^2) [$^*p < 0.05$].

repair-only group ($p = 0.08$, 28% increase). The stiffness and toughness of TGF-β3-treated tendons were also increased compared to carrier controls (stiffness: $p = 0.04$, 50% increase compared to experimental group carrier; toughness: $p = 0.007$, 34% decrease compared to control group carrier, Fig. 5B,E). No differences were found in the mechanical properties (ultimate stress and modulus) when comparing the TGF-β3-treated tendons to all other groups at this early timepoint. At 56 days, the tendons in the TGF-β3-treated group had increased ultimate stress compared to tendons in the carrier-only groups, but these differences were not significant ($p = 0.08$, 48% increase compared to control group carrier; $p = 0.12$, 41% increase compared to experimental group carrier, Fig. 5C). A significant increase was seen for modulus when comparing the TGF-β3-treated group to the experimental group carriers ($p = 0.01$, 37% increase, Fig. 5D). The toughness of the TGF-β3-treated group was also significantly increased over the repair-only group ($p = 0.03$, 36% increase, Fig. 5E). Changes over time were observed in ultimate load and toughness. The ultimate load increased over time in all groups ($p = 0.07$, 22% increase for TGF-β3 group, $p < 0.05$, 31–46% increases for all other groups). Similar significant trends were seen for toughness over time ($p = 0.001$). Stiffness, ultimate stress, modulus, and toughness were significantly lower

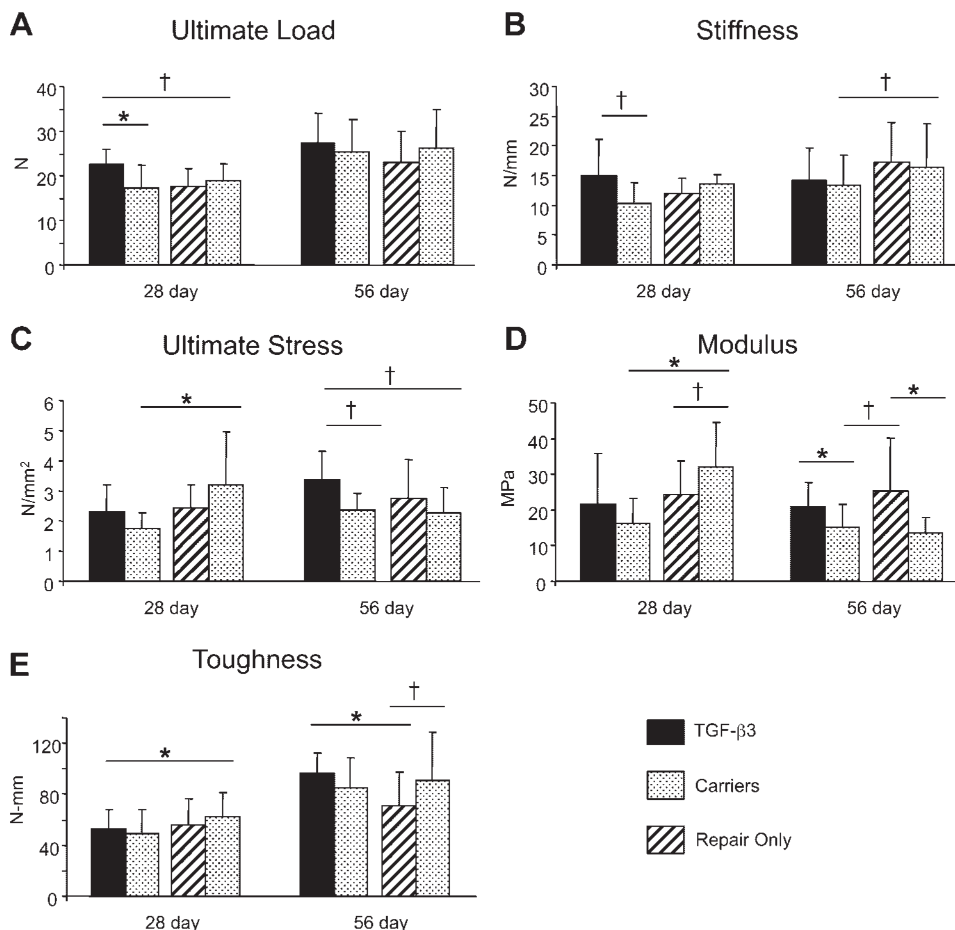

Figure 5. Biomechanics. At 28 days, the structural properties (ultimate load (A), stiffness (B), and toughness (E)) of the TGF-β3-treated insertions were increased compared to carrier controls (i.e., experimental group carriers and control group carriers). Ultimate load increased in all groups over time, approaching normal/uninjured values (normal = 28.8 ± 6.0 N). At 56 days, the mechanical properties (ultimate stress (C) and modulus (D)) of the TGF-β3-treated insertions were increased compared to carrier controls. Stiffness, ultimate stress, modulus, and toughness of injury and repair groups did not reach normal values by 56 days (normal values: stiffness = 37.8 ± 8.8 N/mm, ultimate stress = 11.1 ± 2.7 MPa, modulus = 128.3 ± 40.6 MPa, toughness = 43.1 ± 12.9 N/mm) [$^*p < 0.05$, $^\dagger p < 0.1$].

in all injury and repair groups at 28 and 56 days compared to the normal/uninjured group ($p < 0.01$ for all groups). Ultimate load, however, was not significantly different when comparing the normal/uninjured group to most injury and repair groups at 56 days ($p = 0.68$, 4% decrease for TGF-β3 group; $p = 0.22$, 12% decrease for experimental group carrier; $p = 0.37$, 9% decrease for control group carrier). The one exception was the repair-only group, in which ultimate load remained significantly lower than normal at 56 days ($p = 0.05$, 20% decrease).

DISCUSSION

We expected that sustained delivery of TGF-β3 would reduce the amount of fibrovascular scar tissue formed during adult tendon-to-bone healing, thus leading to an improvement in the structural and material properties of the healing tendon-to-bone insertion compared to untreated controls. Contrary to our hypothesis, delivery of TGF-β3 did not lead to a reduction in scar tissue formation. Instead of promoting regeneration of a fibrocartilaginous transition between tendon and bone, TGF-β3, led to an increase in disorganized scar tissue. The lack of regenerative healing with TGF-β3 may have been due to differences between adult and postnatal environments. For example, the cell types present (and their pluripotency) are quite different prenatally compared to postnatally. The response of a mesenchymal stem cell to TGF-β3 will be much different than the response of a primary fibroblast to TGF-β3. Further studies are required to determine why TGF-β3 did not have the desired effect in adult tendon-to-bone healing.

As seen by the increase in cross-sectional area at the early time studied, TGF-β3 promoted an increase in extracellular matrix formation during healing. Although TGF-β3 delivery did not lead to a reduction in scar formation, it did lead to a significant improvement in structural and mechanical properties of the healing tendon-to-bone insertions. At the early timepoint, ultimate force and stiffness were increased in the TGF-β3-treated insertions compared to the untreated control insertions. At the later timepoint, ultimate stress and modulus were increased in the TGF-β3-treated insertions compared to untreated control insertions. These results indicate that TGF-β3 treatment accelerated all phases of the healing process: inflammation was enhanced at early times (seen histologically), cell proliferation and matrix synthesis were increased at early times (seen histologically, geometrically, and structurally), and extracellular matrix remodeling was increased at later times (seen histologically and in mechanical properties). Thus, treatment with TGF-β3 led to increases in cross-sectional area and a resultant increase in structural properties at early timepoints. With time, tissue remodeling led to a decrease in cross-sectional area and an increase in mechanical properties (a better "quality" tissue). These results were most apparent for toughness, but improvements were also seen in modulus and failure stress.

Implantation of the delivery system alone (without growth factor) had no significant effect on healing. The biomechanical and histological properties of the insertions that received the carrier alone did not differ significantly from the insertions that received surgical repair only. Thus, differences seen in the TGF-β3-treated group can be attributed to TGF-β3 itself and not to any components of the delivery system. Differences between the two carrier groups (i.e., experimental group carriers and control group carriers) however were evident in some biomechanical properties. For example, ultimate stress and modulus at 28 days were significantly higher in the control group carriers compared to the experimental group carriers. The only difference between these two groups was the treatment in the contralateral shoulders. For the control group carriers, the contralateral shoulder received surgical repair only. For the experimental group carriers, the contralateral shoulder received TGF-β3 treatment. Some systemic effects may have resulted from the local delivery of TGF-β3.

The rat is an appropriate animal model for studying the rotator cuff based on anatomic considerations.[16] Rats have a coraco-acromial arch similar to that of humans, under which the supraspinatus tendon passes repeatedly during shoulder motion. This arch is notably missing in most nonprimate larger animal species. Moreover, the rat rotator cuff is sufficiently large to perform a repair that is technically similar to that used clinically. However, larger animals are better suited to reproduce the more complex surgical techniques currently used. A limitation of our injury and repair model is that it approximates an acute rotator cuff injury and does not represent the chronic degenerative rotator cuff tears seen in the majority of clinical patients. However, while our results cannot be applied to the chronically degenerated rotator cuff, they are relevant to the human shoulder and to acute tendon injuries. A possible limitation in this study is the use of bilateral surgeries. However, our experience with bilateral surgeries in past studies has shown no differences in left versus right shoulder activity based on behavioral observations.

Two additional limitations apply to our study. First, although sustained release of TGF-β3 from the HBDS was demonstrated in vitro, the in vivo release kinetics are unknown and likely differ from that of the in vitro environment in absolute terms. Second, the mechanism behind the improved mechanical properties we observed in the TGF-β3-treated tendons remains unknown. Improvements in tissue biomechanics may have resulted from differences in matrix production (e.g., collagen III/collagen I ratio), collagen cross-linking collagen orientation, growth factor expression, cell migration, and cell recruitment.

In summary, we demonstrated that TGF-β3 can be delivered at a sustained rate using a fibrin matrix containing a HBDS and that use of this delivery system in vivo enhances tendon-to-bone healing in a rat model.

Although TGF-β3 treatment led to improved toughness relative to repairs that received conventional treatment (i.e., surgical repair only), the biomechanical properties of TGF-β3-treated tendons were still significantly inferior to those of normal (uninjured) tendon-to-bone insertions. Thus, while delivery of TGF-β3 for enhanced tendon-to-bone healing holds promise, further studies are necessary to optimize the delivery methods and the choice of biological factors for improved healing.

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