Smad8/BMP2–Engineered Mesenchymal Stem Cells Induce Accelerated Recovery of the Biomechanical Properties of the Achilles Tendon

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Summary

Tendon tissue regeneration is an important goal for orthopedic medicine. We hypothesized that implantation of Smad8/BMP2–engineered MSCs in a full-thickness defect of the Achilles tendon (AT) would induce regeneration of tissue with improved biomechanical properties. A 2 mm defect was created in the distal region of murine ATs. The injured tendons were then sutured together or given implants of genetically engineered MSCs (GE group), nonengineered MSCs (CH3 group), or fibrin gel containing no cells (FG group). Three weeks later the mice were killed, and their healing tendons were excised and processed for histological or biomechanical analysis. A biomechanical analysis showed that tendons that received implants of genetically engineered MSCs had the highest effective stiffness (> 70% greater than natural healing, p < 0.001) and elastic modulus. There were no significant differences in either ultimate load or maximum stress among the treatment groups. Histological analysis revealed a tendon-like structure with elongated cells mainly in the GE group. ATs that had been implanted with Smad8/BMP2–engineered stem cells displayed a better material distribution and functional recovery than control groups. While additional study is required to determine long-term effects of GE MSCs on tendon healing, we conclude that genetically engineered MSCs may be a promising therapeutic tool for accelerating short-term functional recovery in the treatment of tendon injuries.

Keywords

Tendon repair; Smad8/BMP2; Tissue engineering; Biomechanics; Achilles tendon

Introduction

Tendon tissue has a highly organized structure of parallel collagen fibers embedded within an extracellular matrix allowing it to withstand and transmit significant forces between muscle and bone. Tendon injuries include tendinitis, tendinosis, bursitis, epicondylitis, and

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complete ligament or tendon rupture. The more common of these injuries are sustained by the supraspinatus tendon of the rotator cuff, the Achilles tendon (AT), and the flexor tendons of the hand (1). In the U.S. at least 200,000 patients undergo tendon or ligament repair each year (2). After tendon injury, the natural healing process results in formation of fibrotic scar, and the structural, organizational, and mechanical properties of the healed tissue are inferior to those of normal tendon (3,4). Although these properties improve over time, they do not return to normal levels (5). Therefore, these injuries present a major clinical challenge.

Therapeutic options consist of autografts, allografts, and synthetic prostheses. None has provided successful long-term repair. Use of allogeneic implants can lead to an immune reaction (6,7), use of autografts can produce donor site comorbidity, and use of synthetic grafts frequently results in degeneration and implant failure (8,9). Biological solutions have also been investigated including use of the growth differentiation factors 5, 6, and 7, which led to ligament and tendon formation in vivo or to regeneration of an injured tendon or ligament (10,11).

Several attempts to use adult mesenchymal stem cells (MSCs) to repair tendon tears were also reported. MSCs can differentiate into mesenchymal phenotypes including osteoblasts, chondrocytes, adipocytes, and muscle- or tendon/ligament–forming cells (12–14). Naïve MSCs generate a positive effect on healing of tendon defects, with improved biomechanical and histological properties of regenerated tissues (15–19). Genetically engineered MSCs were also used for tendon regeneration; however, this resulted in limited improvement compared to treatment with naïve cells (20) or regeneration in a partial-defect model, but no biomechanical analysis was performed (21).

C3H10T1/2 is a murine MSC line that mimics biological properties of human adult MSCs and readily differentiates into bone or cartilage tissue following overexpression of BMP2 or T-box factor (22, 23). C3H10T1/2 cells were used in studies as a model for adult MSCs (21–30). Although these cells were derived from an embryonic mesenchymal cell line, they do not form teratomas upon ectopic implantation and can be manipulated to form the characteristic tri-linage differentiation (osteogenic, chondrogenic, and adipogenic). But C3H10T1/2 cells are a model of adult stem cells and thus possible differences between this cell line and primary cells should be considered. Previously, we engineered this cell line to coexpress BMP2 and a biologically active truncated form of the Smad8 intracellular protein, which is critical for transmitting signals from cell-surface receptors of TGF-β/BMP superfamilies to the nucleus. We used the genetically engineered cell line to generate new tendon formation in vitro and in vivo in a rat AT partial-defect model (21). Although expression of BMP2 in C3H10T1/2 cells induces osteogenesis, we observed that coexpression of the biologically active truncated Smad8 and BMP2 induced new tendon formation and blocked differentiation of these cells into cartilage and bone tissues. Mere expression of truncated Smad8 cDNA in human MSCs is sufficient to induce new tendon formation in ectopic implantation sites (31). Yet so far no one has evaluated the effect of Smad8/BMP2–engineered MSCs on biomechanical properties of a tendon following a full-thickness tear, including comparing healing induced by naïve MSCs with healing induced by genetically engineered cells. We hypothesized that the use of Smad8/BMP2–modified MSCs would increase tendon healing and that this effect would be reflected in the biomechanical parameters of the tendons.

Scaffolds for stem cell therapies should provide sufficient mechanical support to withstand in vivo forces (32). For soft tissues, such a biomaterial could be a synthetic polymer, copolymer, or natural polymer (33–35). Fibrin is a biopolymer widely used clinically (36). Its advantages include its high seeding efficiency, ability to facilitate uniform cell distribution (37), and adhesion capability (32). Fibrin can be produced from the patient’s
own blood, thus providing an autologous scaffold without risk of foreign body reaction (34). However, fibrin provides no mechanical support; this must be provided by other means such as sutures. Fibrin is a promising scaffold for MSCs in tendon repair [17,38], so we selected fibrin as our scaffold.

We performed biomechanical tests of tendon failure load/stress and tendon stiffness/modulus, functional endpoints commonly employed to judge healing efficacy (17,39), and H&E staining of histological sections to estimate the structure of newly formed tissue (40).

Materials and Methods

Cell Culture

Genetically engineered MSCs (from the C3H10T1/2 MSC line, which overexpresses the Smad8 and BMP2 genes) were generated as previously reported (21). These cells and nonengineered MSCs, were suspended in Dulbecco modified Eagle medium (DMEM) that was supplemented with 2 mM L-glutamine, 100 U/ml penicillin, 100 U/ml streptomycin, and 10% fetal calf serum (Biological Industries, Kibbutz Beit Haemek, Israel). The cells were then plated onto tissue-culture dishes at a density of $1.5 \times 10^5$ cells/100-mm culture plate at 37°C in an atmosphere of 5% CO₂/95% air. The medium was changed first after 72 hrs and thereafter every 3 to 4 days. The cells were cultured until confluence, after which they were detached by incubation with 0.25% trypsin–EDTA. Aliquots of $10^6$ cells were prepared and washed twice in phosphate-buffered saline (PBS). Some of the cells were labeled with Vybrant CM-DiI cell-labeling solution (Molecular Probes Inc., Eugene, OR). Labeling was performed by resuspending the cells in serum-free DMEM at a concentration of $10^6$ cells/ml, mixing the cell suspension with Vybrant DiI solution (7 μl/ml cell suspension), and incubating the cells in the dark for 25 mins at 37°C in an atmosphere of 5% CO₂/95% air. The DiI-labeled MSCs were subsequently washed twice with PBS. The cells were immediately implanted in an AT full defect or in a subcutaneous (SC) site in female C3H/HeN mice.

Animal Surgery

The Hebrew University of Jerusalem Institutional Animal Care and Use Committee approved all procedures. A full-thickness AT defect was created in C3H/HeN mice (Fig. 1). 40 female mice, 8–10 wks of age, were anesthetized using a ketamine-xylazine mixture (75 mg/kg ketamine and 10 mg/kg xylazine injected intraperitoneally). Each mouse received an intraperitoneal injection of carprofen (5 mg/kg Rimadyl, Vericore Ltd., Marlow, UK) to reduce postoperative pain or inflammatory response. The skin was shaved and swabbed with chlorhexidine gluconate (0.5%). The AT was separated from the plantaris and soleus tendons by using a spatula and rinsed with PBS. The tendon was sutured around the defect site, and a 2-mm full-thickness defect was created in the distal region of the tendon (Fig. 1). 5 groups were used: 1) the genetically engineered MSC group (GE group), in which $10^6$ genetically engineered MSCs suspended in fibrin gel (1 μl thrombin [10 IU/ml] and 4 μl fibrinogen [46 mg/ml]) (Tisseel kit, Baxter) were implanted in the defect site and in an SC site (n = 10); 2) the non-engineered MSC group (CH3 group), in which $10^6$ nonengineered MSCs (C3H cells) suspended in fibrin gel were implanted in the defect site (n = 10); 3) the fibrin gel–only group (FG group), in which PBS was used instead of cells (n = 10); 4) the natural healing group (NH group), in which the tendon defects were only sutured (n = 10); and 5) the IT control group, a group consisting of intact tendons from the contralateral untreated hind limb of each mouse (n = 40). The skin was closed using 2-0 Mersilk suture, and the animals were returned to microisolator housing. Following surgery, the mice were allowed to move immediately, and thus tension on their tendons returned to about normal. Three weeks after surgery the mice were killed by administration of pental (2 μl/g body
weight; CTS Chemical Industries, Kiryat Malachy, Israel). This time point was chosen due to the fact that accelerated healing has important clinical relevance in terms of accelerated return to load-bearing and reduced time in rehabilitation. Moreover, cross-sectional time point studies of murine AT healing (39) and our own pilot studies indicated that healing trends at 6 wks matched those at 3 wks. The ATs were excised and processed for further use. Samples designated for histology were kept in a 4% formalin solution; samples designated for biomechanical analyses were wrapped with saline-soaked gauze and kept at 20°C until use.

Biomechanical Analysis

The biomechanical analysis was performed as previously described (41,42). In brief, ATs were dissected leaving the calcaneus and gastrocnemius and soleus muscle insertions intact. The tendon sheaths were also maintained to preserve the natural anatomical structure and relative orientation of individual tendon bundles. Muscle fibers were carefully removed to expose the intramuscular tendon, which was bonded to paper by using cyanoacrylate adhesive, leaving ~6–8 mm of exposed tendon midway between the calcaneus and the bound intramuscular fibers. The repaired tendons had an approximately elliptical cross-section. Mechanical tests were performed using a universal testing machine (Zwick 1456, Ulm, Germany) outfitted with a 50-N load cell. Specimens were clamped for testing, with the calcaneus mounted at 30° dorsiflexion to approximate neutral anatomical position. The tendons were tested in saline at 35°C at a strain rate of 0.005/s. Gage length (tendon length from the muscle insertion to bone insertion) was established at a preload of 0.1 N, and the tendons were preconditioned with 10 cycles of 0–10% nominal strain before they were loaded to failure.

Stiffness, ultimate load, and strain at ultimate load were determined. Machine based strains were used in determining biomechanical performance; our previous studies indicated no slippage at the clamp interfaces with reproducible machine strains that strongly correlated to optically measured strains in the tendon midsubstance (R=0.8, p < 0.001; (42)). Noncontact measurements of tendon cross-sectional areas (CSAs) were obtained by frontal and lateral imaging at a preload of 0.1 N with telecentric lenses (pixel resolution ~0.015 mm)(43). The CSA was determined from these measurements assuming an elliptical cross-section. Force was then normalized by the CSA to yield nominal stress values. Stress-strain curves were analyzed for maximal stress, strain at failure, and elastic modulus. Forces and displacements (and corresponding stresses and strains) were determined from the linear region of the material curve, generally between 10 and 15% nominal strain (immediately following the “heel” region (44).

Histological Analysis

2 mice/group were selected for histology. The samples were decalcified by incubating them in 0.5 M EDTA in saline (pH 7.2), whereas the ectopic SC implants were directly processed. All samples were dehydrated in a graded series of ethanol, embedded in paraffin, and cut into 50-μm sections. These sections were stained with H&E. Samples containing CM-DiI–labeled cells were counterstained with 1 μg/ml DAPI (Sigma Chemical Co., MO). The resulting tissue stains were analyzed using light and fluorescence microscopes.

Statistical Analysis

A Lilliefors test was used to verify normal data distributions (45). The data were compared using ANOVA to identify presence of differences among groups, and post-hoc t-testing with Holm correction (46) for multiple comparisons was performed to identify between-group differences. A probability value < 0.05 was considered significant.
Results

Biomechanical Analysis

representative force-displacement and stress-strain curves are shown in Figs. 2A and 2B, respectively. Tendons treated with genetically engineered MSCs exhibited the best healing as defined by highest overall effective stiffness (Fig. 3). Using healthy tendons from the IT control group (11.5±3.2 N/m) as the standard (100% stiffness), tendons in the GE group recouped 85% of their initial stiffness (9.7±1.6 N/m) compared with only 68% in the FG group (7.8±1.9 N/m), 69% in the C3H cell group (7.9±1.6 N/m), and 49% in the NH group (5.6±2.3 N/m). All groups were significantly less stiff than intact controls (p<0.001), with significantly higher stiffness in GE compared to NH (p=0.001), FG (p=0.03), and C3H (p=0.02). CSA measurements showed indistinguishable values for the NH, FG, and GE groups (1.35±0.22, 1.31±0.4, and 1.37±0.39 mm², respectively); The C3H group had higher values (1.69±0.23 mm², p=0.025) (Fig. 4A). All of the treated groups had significantly higher CSAs than the IT control group (0.58±0.14 mm², p<0.001).

The elastic modulus was significantly greater in the GE group than in the NH and C3H groups (58.2±20.2 MPa in the GE group compared with 35.6±0.0 MPa in the NH group and 36.4±8.0 MPa in the C3H group, Fig. 4B). Compared with the GE group, the elastic moduli were similar in the FG group (50.4±23.5 MPa) and much higher in the IT control group (393±135MPa) (data not shown). Elastic modulus represents a material property of tissue; therefore, with its small CSA, the GE group is likely to have less scarring than other groups. Nevertheless all treated groups’ elastic moduli remained below that of the IT control group.

Groups were similar in ultimate load bearing and ultimate stress (Figs. 5A–B). Similar failure modes were observed in all treatment groups (the majority failing by avulsion at the tendon-bone insertion or at the muscle insertion; Fig. 5C). Few samples failed in the tendon midsubstance for any treatment group. While gage length of all treatment groups were 15–35% longer than control tendons (p=0.03), the lack of differences between treatment groups indicated similar gap formation regardless of the applied treatment (Fig. 6).

Histological Analysis

Native tendon was rich in dense, highly organized collagen fibers oriented parallel to each other and with small fibroblasts located between them (Fig. 7A). Sections from the NH group demonstrated poorly organized, newly repaired tissue (Fig. 7B). The tendon structure in the FG group displayed more organization, with dense round cells (Fig. 7C). In the GE group the cells appeared organized in parallel columns, with a more structured ECM and few spindle-shaped cells (Fig. 7D), as previously reported (21). We found no evidence of other cellular phenotypes in GE tissue. The implanted genetically engineered MSCs were also detected by fluorescent labeling in the defect site (Fig. 7E). In sites implanted with nongenetically engineered MSCs (C3H cells), we found small round cells surrounded by a dense ECM but with less collagen fiber organization (Fig. 7F) compared with the other groups. These observations are agree with a previously published analysis of healed tendon in which bone marrow–derived stem cells were used (40). Subcutaneous implants of genetically engineered MSCs demonstrated similar morphological characteristics to that of new tendon tissue formation, as previously reported (21) (data not shown).

Discussion

Two main approaches for tendon repair using MSCs have been pursued. In the first, naïve MSCs are used to induce regeneration. Awad and coauthors (16) isolated MSCs from rabbit bone marrow, placed them in collagen type I gel, and implanted both into a defect in the rabbit patellar tendon. Four weeks after surgery, the cells produced a positive effect on
biomechanical properties (compared to untreated defects), but no visible improvement in microstructure was noted at the defect site. When this experiment was repeated using a prolonged healing time, improvements in biomechanical properties were identified as well as a microstructure identical to that of natural healing controls. Despite these positive findings, bone formation developed in 28% of the implanted sites (15). In a similar study using a collagen sponge carrier, even better results were obtained; tendons implanted with cell-loaded sponges exhibited 60% of native tendon maximum force, 50% of maximum stress, and 75% of linear stiffness. Moreover, at 12 wks the histological appearance of cell-implanted defects was identical to that of healthy controls (18). Similar results were obtained by Young and coauthors (19) when they implanted naïve rabbit bone marrow–derived MSCs into a defect created in the rabbit AT, while Chong and coauthors (17) demonstrated that cell transplantation in an AT defect model in rabbits resulted in improved biomechanical and morphological parameters during the early stages of healing. These findings demonstrate the positive effect of MSCs on tendon tear repair. Investigators analyzed the effect of connective-tissue progenitor cell implantation on the repair of tendon defects in a mouse model by using cells derived from human embryonic stem cells (47). Despite the fact that some of the morphological, histological, and functional properties of the injured tissue were restored after cell implantation, no improvement in mechanical properties was shown.

The second cell-based approach to achieve tendon regeneration focuses on the use of genetically modified MSCs. Schnabel and colleagues (20) used MSCs that overexpressed insulin-like growth factor 1 (IFG-1) to heal collagenase-induced bilateral lesions causing tendinitis in equine flexor digitorum superficialis tendons. Although implantation of either engineered and naïve MSCs in the defect site resulted in improved histological scores, there was no improvement in the biomechanical properties of implanted compared with natural healing tendons. We transfected BMP2–expressing MSCs with a biologically active Smad8 variant and analyzed the effect of this gene combination on the cells’ ability to differentiate into tendon-like tissue (21). The cells could regenerate tendon tissue in vivo and repair a partial AT defect by collagen formation. However, at that time we did not test the biomechanical properties of the repair. Moreover, the model was a partial-thickness rather than a full-thickness defect. Those results were reproduced using genetically engineered human MSCs; both tendon and osteotendinous junction formation occurred when these cells were implanted ectopically (31).

Our results indicate that Smad8/BMP2–engineered MSCs implanted in a tendon defect site remain viable and accelerate early recovery of biomechanical properties. Nonengineered MSCs suspended in fibrin gel also accelerate the biomechanical recovery of ATs, as reported in an earlier study (17). In the present study, injured tendons that received genetically engineered MSCs displayed the best healing in terms of effective stiffness. This finding is important because stiffness is the most clinically relevant structural property in tendons and ligaments (48–50).

Tendons repaired using genetically engineered MSCs had marginally improved material quality compared to controls (higher elastic modulus and a tendency toward lower CSAs). A higher modulus reflects more efficient material distribution; the higher modulus in the GE MSC group was partly supported by histology indicating that tendons receiving genetically engineered MSCs showed reduced scarring compared to other groups. The CSA in all treatment groups remained nearly double that of intact tendons, indicating that the healing process was ongoing and still relatively far from scarless.

The NH group recovered stiffness much more quickly than previously documented (39), indicating that the C3H/HeN strain may have a better healing capacity than the A/J strain.
used in that study. Thus, testing of Smad8/BMP2–engineered MSCs in a different animal model that does not heal as fast as the C3H/HeN mouse is required to study the long-term effect of engineered MSC implantation on the biomechanical properties of repaired tissue. Finally, it seems that fibrin gel has some beneficial effects on tendon healing and should be considered for further applications in tendon therapy.

We chose to perform biomechanical analysis 3 wks post-treatment since accelerated healing has important clinical relevance. We were further motivated by our intention to reduce the number of required animals to indicate benefit. Cross-sectional time point studies of murine AT healing (51) and our own pilot studies indicated that while healing trends at later time points matched those at earlier time points relative effect sizes at longer times were smaller and experimental variability was higher. A longer healing point would have necessitated the use of a comparatively large number of animals (~25 per group) to achieve sufficient power.

In summary, we present a relevant clinical approach to tendon healing. This appears to be the first time that such an acceleration in restoring effective stiffness has been shown in an AT full-thickness defect model. Additional studies are required to determine the effect of genetically engineered MSCs on tendon repair at later time points after surgery. Such studies should include tendon tear models in larger animals and in additional sites such as the rotator cuff tendon.

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**References**


Figure 1.
Tendon defect. A. A full-thickness defect was created in the AT of C3H/HeN mice (arrow). B. Cells suspended in fibrin gel were subsequently implanted in the defect site (arrow).
Figure 2.
Representative test curves from treated tendons. A. Force displacement curves for specimens with median stiffness. B. Corresponding curves normalized to stress and strain.
Figure 3.
Stiffness of the injured AT in 4 groups: C3H = AT received nonengineered cells; FG = AT received fibrin gel with no cells; GE = AT received genetically engineered cells; and NH = natural healing of AT. The probability values were < 0.05 for a comparison of the FG and GE groups and a comparison of the C3H and GE groups. The probability value was 0.001 for a comparison of the GE and NH groups.
Figure 4.
Material Quality Analysis. A. The NH, FG, and GE groups had similar values of CSAs. B. Elastic modulus was significantly higher in the GE group than in the NH and C3H groups ($p < 0.05$). The modulus in the FG group was similar to that in the GE group, and the value in the group of intact tendons (IT control group) was much higher (data not shown). C3H = AT received nonengineered cells; FG = AT received fibrin gel with no cells; GE = AT received genetically engineered cells; and NH = natural healing of AT.
Figure 5.
Ultimate Strength and Failure Mode. A. No statistical differences in load-bearing capacity were observed between groups. B. Ultimate stress values in all treatment groups were significantly different from uninjured controls (native, p<0.01). No differences in load-bearing capacity were observed between treatment groups. C. No significant differences in failure mode were observed; the majority of tendons in each group (except C3H) failed by avulsion at the tendon insertion to bone. C3H = AT received nonengineered cells; FG = AT received fibrin gel with no cells; GE = AT received genetically engineered cells; and NH = natural healing of AT.
Figure 6.
Gap formation differences in treated muscle-to-bone insertion length at pre-load compared to native tendon ($p<0.05$), indicating formation of a gap of between 1 and 2 mm in treated tendons. No significant differences were observed between treatment groups.
Figure 7.
A. Intact tendon (IT control group) displayed highly organized dense collagen fibers oriented parallel to each other with cells assembled between the fibers. B. Tendon allowed to naturally heal (NH group) had poorly organized new tissue. C. Tendon treated with fibrin gel alone (FG group) had an organized structure with round cells. D. Tendon treated with genetically engineered MSCs (GE group) had an organized structure of parallel bundles of extracellular matrix and spindle-shaped cells. E. CM-DiI imaging identified genetically engineered MSCs in the implantation site 3 weeks after treatment. F. Tendon that received nonengineered MSCs (CH3 group) had small round cells that were not highly organized, as well as an abundance of ECM.