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Multilayer Tendon Slices Seeded with Bone Marrow Stromal Cells: A Novel Composite for Tendon Engineering

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Abstract

The ideal scaffold for tendon engineering would possess the basic structure of the tendon, native extracellular matrix, and capability of cell seeding. The purpose of this study was to assess the tissue engineering potential of a novel composite consisting of a decellularized multilayer sliced tendon (MST) scaffold seeded with bone marrow stromal cells (BMSC). BMSC and infraspinatus tendons were harvested from 20 dogs. The tendons were sectioned in longitudinal slices with a thickness of 50 μm . The slices were decellularized, seeded with BMSC, and then bundled into one composite. The composite was incubated in culture media for 14 days. The resulting BMSC-seeded MST was evaluated by qRT-PCR and histology. The BMSC viability was assessed by a fluorescent tracking marker. Histology showed that the seeded cells aligned between the collagen fibers of the tendon slices. Analysis by qRT-PCR showed higher tenomodulin and MMP13 expression and lower collagen type I expression in the composite than in the BMSC before seeding. BMSC labeled with fluorescent tracking marker were observed in the composite after culture. Mechanical testing showed no differences between scaffolds with or without BMSC. BMSC can survive in a MST scaffold. The increased tenomodulin expression suggests that BMSC might express a tendon phenotype in this environment. This new composite might be useful as a model of tendon tissue engineering.

Keywords

bone marrow stromal cells; tendon; regeneration; gene expression; biomechanics

Tendon injuries are very common and place a large burden on the US economy. It has been estimated that more than 32 million patients sustain traumatic and overuse injuries to tendons and ligaments, at a cost of roughly \$30 billion per year.^{1,2} Functional restoration of the injured tendon is still a great challenge. Tissue engineering approaches provide the possibility to enhance tendon healing or regenerate tendon tissue. Despite recent advances,^{3,4} the technology necessary to achieve the goal of generating a mechanical, biological, and functional tendon is still in its infancy.

The three factors necessary for tissue regeneration are cells, scaffolds, and growth factors.⁵ Various kinds of artificial porous or gel scaffolds have been developed for bone and cartilage

regeneration.^{6–9} For tendon regeneration, the ideal scaffold would possess: mechanical properties similar to normal tendon; the basic structure of the tendon, in which type I collagen fibers align densely in the same direction; cells expressing a tendon phenotype, and compatibility with surrounding tissues. The scaffold should also provide an appropriate environment, such as interconnected cavities, for cells or growth factors to invade the scaffold and for the ingrowth of adjacent tissue. Various synthetic scaffolds and a cellular scaffolds have been developed for tissue engineering. However, they might have problems in tissue regeneration or healing.^{10–13}

Cell-seeded collagen sponges or gels have been used for tendon engineering and have been shown to be useful in animal studies,^{2,14} but such methods do have some relative disadvantages, such as the bioreactors and chemical or cytokine stimulations required to achieve the aligned collagen fibers and tenocyte differentiation in vitro. Secondly, the mechanical strength of collagen scaffolds is too low to withstand mechanical stress over time.¹⁴ Polyesters, such as polyglycolic acid (PGA), polylactic acid (PLA), and polylactic polyglycolic acid (PLGA) are common polymers for tendon tissue engineering.¹⁵ However, their hydrophobic nature does not support a high level of cell adhesion.^{16,17} Also, although those polymers have the benefit of being natural metabolites, they are also acidic, which can give rise to significant systemic or local reaction.^{18,19} In contrast, native decellularized fresh tendon tissue is in many ways an ideal scaffold environment for cell seeding, adhesion, and survival, containing native collagen, with a normal tendon structure and organization.

The purpose of this study was to assess the mechanical properties of a multilayer composite which might serve as a scaffold for tendon regeneration, the viability of bone marrow stromal cells (BMSC) seeded into the composite, and the differentiation potential of the BMSC within the composite, using functional markers. We hypothesized that BMSC could successfully populate the a cellular composite and remain viable in tissue culture. We also hypothesized that the BMSC-seeded scaffold would increase the maximum failure load and stiffness of the composite. Finally, we hypothesized that the seeded BMSC would express genes related to the tendon phenotype.

MATERIALS AND METHODS

Bone Marrow Stromal Cells Harvest

Twenty mixed-breed dogs weighing between 25 and 30 kg were used for bone marrow and tendon tissue harvesting. These dogs were being euthanized for other Institutional Animal Care and Use Committee (IACUC)-approved studies. Immediately prior to sacrifice, the dogs were anesthetized with intravenous ketamine (13 mg/kg) and diazepam (6 mg/kg), and maintained under anesthesia with 1.5% isoflurane. A total of 4.0 ml of bone marrow was aspirated from the medial aspect of the proximal tibiae using an 18-G needle and 20-ml syringe (BD, Franklin Lakes, NJ) containing 1.0 ml of heparin solution (Heparin sodium injection, Baxter Healthcare Corporation, Deerfield, IL). Then, the dogs were euthanized by an overdose of pentobarbital, and the infraspinatus tendons of both shoulders were harvested. The infraspinatus tendon was exposed by removing the deltoid muscle, and the tendinous portion between the insertion to the bone and the muscle-tendon junction was harvested. The size of harvested tendon was roughly 25 × 10 mm, and rectangular in shape.

The harvested tendons were frozen at -80°C until processing. Both the bone marrow and infraspinatus tendons were harvested under sterile conditions. The donor of the BMSC was not matched with the donor of the tendon in making the composite of BMSC and tendon slices for this in vitro experiment.

The heparinized bone marrow extract was added to 5.0 ml phosphate buffered saline (PBS), and centrifuged at 1500 rpm (380 g) for 5 min at room temperature. The bone marrow pellet was resuspended in 10 ml of minimal essential medium (MEM) with Earle's salts (GIBCO, Grand Island, NY), 10% fetal bovine serum (GIBCO), and 5% antibiotics (Antibiotic-Antimycotic, GIBCO). The cells from one dog were divided into four equal aliquots, placed in 100-mm culture dishes and incubated at 37°C with 5% CO_2 and 95% air at 100% humidity. After 5 days, the medium and any floating cells were removed, and new medium was added to the remaining adherent cells. These adherent cells were defined as BMSC.²⁰ The medium was then changed every other day until the adherent cells reached confluence. The cells were then released with trypsin-EDTA solution (0.25% trypsin, 0.1% EDTA in HBSS; Mediatech Inc., Manassas, VA) to produce a cell suspension, and centrifuged at 1,500 rpm for 5 min to remove the trypsin-EDTA solution. The concentrated cell suspension from each dog was then gathered in one tube. The cells were counted with a hemacytometer, and the concentration of the cell suspension was adjusted to 5.0×10^6 cells/ml by adding additional medium.

Multilayer Sliced A cellular Tendon Scaffold

Frozen infraspinatus tendon was thawed at room temperature. The infraspinatus tendons were trimmed into segments roughly 25×10 mm in size. The tendon segments were immersed in liquid nitrogen for 2 min and then thawed in saline solution at 37°C for 10 min. This procedure was repeated five times.^{21,22} Following washing in PBS without EDTA (3×30 min), the tendon segments were incubated in 20 ml of nuclease solution from bovine pancreas, 1.5 Units/ml (Roche Diagnostic, Indianapolis, IN) for 12 h at 37°C . Finally, the infraspinatus tendon segments were rinsed for 30 min in PBS (50 ml) at room temperature with gentle agitation. The rinsing was repeated three times. The tendon segments were then frozen to -80°C and fixed to the cutting base plate of a cryostat (Leica CM1850, Nussloch, Germany) with O.C.T. compound (polyvinyl alcohol and polyethylene glycol; Tissue-Tek®, Sakura Finetek USA, Inc., Torrance, CA). The excess O.C.T. compound around the tendon was removed by a scalpel. The tendon segments were then sliced at a thickness of 50 μm and the slices were placed in a 100-mm culture dish. Ten slices were placed on each dish. The slices were thawed on the dish in an incubator at 37°C with 5% CO_2 and 95% air at 100% humidity for 10 min. The tendon slices were then washed three times with 10 ml of PBS. At this point, the sliced a cellular tendon segments were ready to seed with BMSC.

Composite of BMSC and Sliced Tendon Scaffold

The concentrated BMSC solution (5.0×10^6 cells/ml, 10 ml/dish) was added to the sliced tendon scaffold dish and cultured at 37°C with 5% CO_2 and 95% air at 100% humidity for 2 days. The slices were then carefully detached with forceps and bundled together on a new dish. The ends of the bundled slices were fixed with 3-0 Ethilon suture (nylon suture; Ethicon Inc., Piscataway, NJ), and a 1.2-g sterilized stainless steel clip (Alligator clip;

Mueller Electric Company, Cleveland, OH) was attached at one end of the sutured bundle. The composite was then suspended in a 15-ml conical tube and immersed in the same medium described above. The clip served as a weight, preventing the composite from floating in the medium. The composites were incubated for 2, 7, or 14 days. As a control, tendon slices without cells were bundled and maintained in medium for the same time periods. The medium was changed every other day.

Assessment of Cell Viability

BMSC from two dogs were stained with the fluorescent marker PKH26-GL (PKH26 Red Fluorescent Cell Linker Kit for General Cell Membrane Labeling; Sigma, St. Louis, MO) before seeding on the tendon slices, following the manufacturer's instructions. This fluorescent marker has been used for cell tracking in the studies using bone marrow stromal cells.^{15,23} Prior to bundling (i.e., after 2 days in culture), single tendon slices with labeled BMSC were examined by a laser scanning confocal microscope (LSM310; Zeiss, Thornwood, NY) without fixation. For the evaluation of the composites incubated for 7 and 14 days, frozen sections of the composite were made at a thickness of 50 μm , and then examined under the confocal microscope. The fluorescent image of the composite after incubation was combined with the image under the ultraviolet light, with the tendon slices observed as blue under the ultraviolet light.

Histological Assessment

The 7- and 14-day incubation composites from two dogs were used for the histological assessment. The tendon slices without BMSC incubated in the medium for 2 days were also used to evaluate whether any host cells remained after repeated freeze-thaw cycles. These samples were fixed in 10% neutral buffered formalin, embedded in paraffin, and sectioned longitudinally at a thickness of 5 μm . Hematoxylin and eosin (H&E) staining was performed.

Assessment of Gene Expression

A quantitative real-time reverse transcription-polymerase chain reaction (RT-PCR) was performed to measure the gene expression levels of tenomodulin (a marker of tenocyte differentiation²⁴), collagen type I and III, MMP2 (gelatinase), and MMP13 (collagenase).²⁵ RNA was extracted by TRIzol[®] reagent (monophasic solutions of phenol and guanidine isothiocyanate; Invitrogen Corporation, Carlsbad, CA). cDNA was synthesized using 1st Strand cDNA Synthesis Kit (Roche) with random primers. Quantitative RT-PCR was performed on a LightCycler[®] (Roche). The sequences of the primers are shown in Table 1. The expression level was normalized to that of GAPDH. All mRNA expressions were confirmed by Light-Cycler[®] melting curve analysis. Eight samples from each group, including the BMSC solution before seeding, the composites incubated for 2, 7, and 14 days, and the intact infraspinatus tendons were used for assessment of gene expression.

Biomechanical Assessment

The maximum failure load and the linear stiffness of the composite were measured with a custom-made microtester,²⁶ which was composed of a linear servo motor (MX 80 Daedal,

Irwin, PA) and a load cell with the accuracy of 0.01 N (MDB-5, Transducer Techniques, Temecula, CA). The composites with BMSC from eight dogs after 7- and 14-days incubation were used. Both sides of the tendon, at a length of 10 mm, were clamped with a custom-designed holder and 0.1 N of preload was applied (Fig. 1). The composite was distracted at a rate of 0.2 mm/s. This assessment was performed immediately after the end of each culture, and the composites were kept moist with PBS during measurement. The ultimate tensile load and the stiffness of the tendon slice were recorded and analyzed. The ultimate tensile load was defined as the maximum load to break the tendon slice. The stiffness was defined as the slope of the load/displacement curve in the linear region.

Statistics

The results of the maximum failure load and the linear stiffness were analyzed by two-way ANOVA, and the results of the gene expressions were analyzed by the Kruskal–Wallis test and Mann–Whitney test, with Bonferroni post hoc adjustment. We used the nonparametric analysis because the gene expression results had large standard deviations, which are not suitable for parametric analysis. All results with $p < 0.05$ were considered significant.

RESULTS

Cell Viability and Histology

The BMSC labeled with PKH26 were observed as red under the confocal laser microscope (Fig. 2A; red: BMSC). After 2 days' culture, viable BMSCs were observed on single slices before bundling. In the bundled composites, the BMSC were also observed between slices after 7 and 14 days in culture (Fig. 2B; longitudinal image 14-days incubation).

Cells were not observed in the unseeded tendon slices after five freeze–thaw cycles. In the composite of BMSC and tendon slices after 7- and 14-days incubation, histological sections showed most cells existed between the tendon slices (Fig. 2C; 14-days incubation; original magnification, $\times 200$).

Gene Expression

The gene expression data are shown in Figure 3. In the decellularized tendon slices without BMSC, GAPDH and other gene expression were not detected. In contrast, the native infraspinatus (ISP) tendon showed high expression of tenomodulin and type III collagen, with low expression of type I collagen and MMPs. BMSC before seeding showed no detectable tenomodulin or MMP13 but did show high ($2 \times$ GAPDH) expression of type I collagen and MMP2, and moderate ($1 \times$ GAPDH) expression of type III collagen. Day 2 composites showed low levels of expression of tenomodulin, collagen I and II, and MMP2 and 13. By day 7, the expression of MMPs had increased, significantly so for MMP13, as compared to the cultured BMSC alone; 14-day composites showed a similar trend.

Maximum Failure Load and Linear Stiffness

The maximum failure load of the composite with BMSC was 1.85 ± 0.86 N and 2.97 ± 1.83 N at days 7 and 14, respectively. The maximum failure load of the composite without BMSC was 2.13 ± 1.50 N and 2.45 ± 0.76 N at days 7 and 14, respectively. The linear stiffness of

the composite with BMSC was 0.59 ± 0.35 N/mm and 1.14 ± 0.80 N/mm at days 7 and 14, respectively. The linear stiffness of the composite without BMSC was 0.74 ± 0.48 N/mm and 0.82 ± 0.30 N/mm at days 7 and 14, respectively. There was no significant difference between scaffolds with or without BMSC, or by time in culture (Fig. 4).

DISCUSSION

Three-dimensional scaffolds have been used with varying degrees of success in tendon tissue engineering. However, synthetic material may alter tendon mechanical properties, decrease cell viability, lose strength and integrity over time, inhibit tendon ingrowth, increase the inflammatory response, and cause scar hyperplasia around the repair site.^{11–13,27} A cellular scaffolds made from animal tissues or human cadavers, such as small intestinal submucosa^{28,29} and dermal matrix,^{10,30} have also been developed and used in both laboratory and clinical studies. Although many studies have demonstrated that these native materials can serve as a temporary scaffold and provide the environment for infiltration by local cells, concerns persist regarding mechanical strength, healing quality, and adhesion formation.^{31–35}

In this study, we evaluated a composite of a cellular tendon slices seeded with BMSC, and bound multiple slices into a thicker composite. We believe that this multilayer tendon composite is a promising tendon regeneration scaffold, which combines normal tendon collagen architecture with the possibility of improved cellularity within and between the slices. A decellularized tendon allograft combined with BMSC from the host could reduce the immunological reaction associated with a conventional tendon allograft, while the slicing and bundling permit construction of a wide array of tendon substitutes, varying in size and strength, without the need for complex or special equipment.³⁶ The bundled composite should also allow for greater cellular ingrowth than is possible in an intact allograft tendon.

Our cell source, BMSC, was chosen because it has the potential to differentiate into a variety of tissue types.³⁷ This differentiation is dependent on the cellular environment (extracellular matrix), biomechanical stimulation, and biochemical (cytokine) stimulation. We used tenomodulin as a specific tendon phenotype marker.²⁴ Although cell viability was assessed in only two samples, the images clearly showed that BMSC successfully seeded onto decellularized tendon slices. The cells survived after bundling multiple slices together for up to 2 weeks in tissue culture, and can express tenomodulin, a marker of the tendon phenotype.

The tenomodulin expression of the BMSCs before seeding was very low, which might be due to the cell culture environment as well as to the normal behavior of the native BMSCs. While the level of tenomodulin expression of the BMSC composite was lower than that of the native infraspinatus tendon tissue, supplementation of growth factors or mechanical stimulation might further enhance tenomodulin synthesis and BMSC differentiation. We plan to study this possibility in the future.

We selected MMP2 and MMP13 for analysis because these are expressed during tendon remodeling.³⁸ The expression of MMP13 in the composite after 7- and 14-days incubation was higher than that of the BMSC before seeding, which might indicate that BMSC could

induce tendon remodeling. The collagen type I expression was significantly higher in BMSC before seeding, even higher than that in the intact tendon. The reason for this higher collagen type I expression in BMSC was unclear. The lower expression of collagen type I in the incubated composite, compared with that in BMSC before seeding, might indicate that the BMSC was in a catabolic state, but there was no significant difference in collagen synthesis between the BMSC composite and normal tenocytes. However, the tenomodulin expression did not increase after 7- and 14-days incubation, which might indicate that the composite was no longer in an anabolic state with this culture method. To promote an anabolic response to BMSC in vitro, additional stimulation might be required. It is also possible that behavior might differ in vivo.

Although some significant differences in gene expression were detected between experimental and control groups, significant differences in mechanical properties, such as maximum failure load and the linear stiffness, were not obtained. This result can be explained as the former is a function of the implanted cells while the latter is a function of the scaffold. Two weeks in cell culture may be insufficient for substantial remodeling of the tendon substrate by the implanted cells, and so the mechanical properties are, not surprisingly, similar in all the groups. We would anticipate that over longer time frames significant remodeling would occur, and that the material properties would change, especially in vivo under loading. The mechanical behavior with different thickness of tendon slices should also be investigated in the future.

There were several limitations of this study. Only one thickness of tendon slices was studied. Future studies should investigate the optimal thickness of the slices, from the aspects of both cellular integration and strength. Second, the other cytokines or growth factors that regulate the expression of collagen I and II or MMPs, such as TGF- β , were not evaluated. Finally, as this was an in vitro study, the tendon composite remodeling in vivo condition would be different and also the immunological reactions of the tendon slices and the composite of BMSC also require an in vivo model.

CONCLUSIONS

We have developed an engineered tendon substitute using a cellular tendon slices seeded with cultured BMSC. We showed that the BMSC could survive up to 2 weeks in vitro, that they organized along the collagen fibers on the tendon slices, and expressed a marker of tendon phenotype, tenomodulin. This new composite might be useful as a model of tendon tissue engineering. Future studies to enhance the tendon composite mechanical strength, cellularity, and incorporation with host tissues would advance this novel technique towards clinical application.

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Figure 1.
Microtester used for tendon slice mechanical property test.

Figure 2.

(A) BMSC (red color) were observed on one tendon slice detached from dish before bundling, after 2-days incubation. (B) This image is a combination of the a fluorescent red label and a nonspecific blue ultraviolet background. Labeled BMSC (red color) were observed on and between tendon slices (blue color under ultraviolet light) after 14-days incubation. (C) H&E-stained section showed dense collagen fibers of the tendon slices, and cells located between tendon slices after 14-days incubation (original magnification, $\times 200$).

Figure 3. The results of gene expression levels of tenomodulin, collagen type I, collagen type III, MMP2 and MMP13 by qRT-PCR. The expression level was normalized to that of GAPDH ($n = 8$).

Figure 4.
Displacement-load curve ($n = 8$).

Table 1

Sequences of Primers Used in Assessment of Gene Expression

Gene	Sequences of forward/reverse primers
GAPDH	5'-TATGATTCTACCCACGGCAA-3'/5'-CAGTGGACTCCACAACATAC-3'
Collagen type I	5'-TGGTTCTCCTGGCAAAGAT-3'/5'-ATCACCGGGTTCACCTTTA-3'
Collagen type III	5'-ACAGCAGCAAGCTATTGAT-3'/5'-GGACAGTCTAATTCTTGTTCGT-3'
MMP2	5'-AGCTACTTCTTCAAGGGTG-3'/5'-GTGTGCAGAAGGCAATG-3'
MMP13	5'-TACAACTTGTTTCCTTGTCGC-3'/5'-CTGGGCCATAGAGAGACT-3'
Tenomodulin	5'-GATCCCATGCTGGATGAG-3'/5'-TACAAGGCATGATGACACG-3'