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A Comparative Study of the Effects of Growth and Differentiation Factor 5 on Muscle Derived Stem Cells and Bone Marrow Stromal Cells in an *In Vitro* Tendon Healing Model

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Abstract

Purpose—To investigate the ability of muscle derived stem cells (MDSCs) supplemented with growth and differentiation factor-5 (GDF-5) to improve tendon healing in comparison to bone marrow stromal cells (BMSCs), in an *in vitro* tendon culture model.

Methods—Eighty canine flexor digitorum profundus tendons were assigned into 5 groups: 1) repaired tendon without gel patch interposition (no cell group), 2) with BMSC seeded gel patch interposition (BMSC group), 3) with MDSC seeded gel patch interposition (MDSC group), 4) with GDF-5 treated BMSC seeded gel patch interposition (BMSC+GDF-5 group), and 5) with GDF-5 treated MDSC seeded gel patch interposition (MDSC+GDF-5 group). After culturing for 2 or 4 weeks, the failure strength of the healing tendons was measured. The tendons were also evaluated histologically.

Results—The failure strength of the repaired tendon in the MDSC+GDF-5 group was significantly higher than that of the no cell and BMSC groups. The stiffness of the repaired tendons in the MDSC+GDF-5 group was significantly higher than that of the no cell group. Histologically, the implanted cells became incorporated into the original tendon in all 4 cell-seeded groups.

Conclusion—Interposition of a multi-layered GDF-5 and MDSC-seeded collagen gel patch at the repair site enhanced tendon healing compared to a similar patch using BMSC. However, this increase *in vitro* was relatively small. In the clinical setting, differences between MDSC and BMSC may not be substantially different, and it remains to be shown that such methods might enhance the results of an uncomplicated tendon repair clinically.

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Clinical Relevance—MDSC implantation and administration of GDF-5 may improve the outcome of tendon repair.

Keywords

Muscle Derived Stem Cell; Bone Marrow Stromal Cell; Growth Differentiation Factor 5; Flexor Tendon Repair

INTRODUCTION

The functional repair of flexor tendon injury, especially in zone II, remains a challenge for hand surgeons.[1–3] Despite improvements in suture materials,[4–6] suture techniques,[7, 8] and postoperative rehabilitation protocols [9, 10] which have generally resulted in improved clinical outcomes, many complications, such as repair site rupture and adhesion formation, still occur and require improved therapeutic solutions. One of the major complications is related to the slow healing of injured tendons, which requires prolonged protection after repair. This complication results from relative hypocellularity of tendons that slows intrinsic healing and results in the migration of extrinsic cells, which in addition to promoting healing form adhesions; however, blocking these cells may further delay healing and result in failure of the tendon repair.[11] Furthermore, the hypocellular condition worsens after an injury and subsequent surgical repair due to cell death in the zone of injury.[12]

To address this problem, stem-cell-based therapies have been introduced with encouraging outcomes in preclinical evaluations.[13–15] Mesenchymal stem cells have been successfully isolated from bone marrow, adipose tissues, muscle, skin, and tendon. The cells most commonly used for tendon repair are bone marrow stromal cells (BMSCs).[16] Indeed, recent work has shown that animal models of cell therapy do promote tendon healing.[13–15] in particular, the use of BMSCs to treat a flexor digitorum profundus laceration injury in a canine *in vitro* model found increased maximal strength and stiffness of the repaired tendons treated with BMSC.[16] Subsequent *in vitro* work showed that addition of platelet rich plasma to the bone marrow stem cell treatment further improved tendon healing.[17] Furthermore, when stem cells were combined with growth differentiation factor – 5 (GDF-5), maximal strength of repaired tendons was enhanced in the same *in vitro* canine flexor digitorum profundus laceration model, while this effect was not seen with either stem cells or GDF-5 treatment alone.[18]

The use of muscle derived stem cells has been evaluated in stem cell mediated tendon healing. The MDSCs can differentiate into multiple cell types, and application of such cells for treatment of cardiovascular, urological, and musculoskeletal disorders has been investigated. Sassoon et al evaluated BMSCs and muscle derived stem cells (MDSCs) for their propensity to differentiate into tenocyte-like cells.[19] The MDSCs showed an increased propensity, compared to BMSCs, toward tenocyte phenotype with increases in tenomodulin, collagen I, and collagen III expression as in cell culture, suggesting that muscle derived stem cells may have an advantage as a cell source for tendon regeneration and healing.

Multiple cytokines, including transforming growth factor (TGF- β), basic fibroblast growth factor, platelet derived growth factor, insulin like growth factor, epidermal growth factor, and vascular endothelial growth factor [20–24] stimulate cell activity and enhance tendon healing. In particular GDF-5 also has been proposed to enhance tendon healing. The GDF-5 is a member of TGF- β superfamily is also known as bone morphogenetic protein (BMP) 14. [25, 26] It has the ability to stimulate BMSC activity and regulate BMSC differentiation to tenocytes [27, 28] and has promoted tendon healing in several animal models.[18, 29–31]

In this study, we hypothesized that interposition of a multi-layered MDSC-seeded collagen gel patch at the repair site would result in higher tendon healing strength than a repair using a BMSC-seeded patch and that introduction of GDF-5 would enhance the effect of stem cell healing. To test this hypothesis, we designed a study in an *in vitro* canine tendon tissue culture model.

METHODS

Study Design

All tissue was harvested at the time of death from mixed-breed dogs that had been involved in other, Institutional Animal Care and Use Committee approved studies and that did not affect the health of bone marrow, paws, or tendons. Bone marrow and vastus lateralis muscle was harvested from 4 dogs. A total of 80 flexor digitorum profundus tendons from the second to fifth hind paw digits of 10 other dogs were harvested under sterile conditions. These tendons were then immediately immersed in cell culture medium consisting of minimal essential medium (MEM) with Earle salts (GIBCO, Grand Island, NY), 10% fetal bovine serum, and 1% antibiotics (Antibiotic-Antimycotic, GIBCO) to maintain tissue viability. The tendons were randomly divided into 5 groups: 1) repaired tendon without gel patch interposition (no cell group), 2) repaired tendon with BMSC-seeded gel patch interposition (BMSC group), 3) repaired tendon with MDSC-seeded gel patch interposition (MDSC group), 4) repaired tendon with GDF-5 treated BMSC-seeded gel patch interposition (BMSC+GDF-5 group), and 5) repaired tendon with GDF-5 treated MDSC-seeded gel interposition (MDSC+GDF-5 group). After 2 or 4 weeks tissue culture, the tendons were assessed biomechanically and histologically.

Bone Marrow Stem Cell Harvest and Suspension

Bone marrow (8.0 mL) was aspirated, under sterile conditions from each tibia with a 12-mL syringe containing 2.0 mL heparin solution. Cells were pelleted, and heparin was removed by centrifugation at 1,500 rpm for 5 minutes at room temperature, and the bone marrow pellet was resuspended in cell culture medium (MEM) with Earle salts (GIBCO), 10% fetal bovine serum, and 1% antibiotics (Antibiotic-Antimycotic, GIBCO) and then plated on 2 100-mm culture dishes in 10 mL of medium, consisting of minimal essential medium. Bone marrow cells were incubated at 37°C with 5% CO₂ and 95% air at 100% humidity. Media were replaced at 3-day intervals. At 70% to 80% confluence, the BMSCs were harvested with 0.25% trypsin-ethylenediaminetetraacetic acid (GIBCO) and expanded. Cells between passage 2 and 4 were used for seeding into the gel patch.

Muscle Derived Stem Cell Harvest and Suspension

The MDSCs were isolated from vastus lateralis muscle by a modified preplate technique [32] that used a series of cell separation based on adherence to isolate the MDSC cell fraction. Specimens were washed in phosphate buffered saline solution and minced with scissors into smaller pieces and enzymatically digested at 37°C with 0.1% type I collagenase (10ml/g tissue)(GIBCO) for 4 hours. After digestion the slurry was passed through a 40-µm nylon filter (BD Biosciences, San Jose, CA) and centrifuged at 1200 rpm for 5 min at room temperature. The cell pellet was washed twice, resuspended in culture media, and plated onto 100-mm culture dishes. Serial preplating was performed to separate cell fractions as described below. Cells were added to 100-mm culture dish. After 30 minutes non-adherent cells in preplate (PP) 1 and medium were transferred to the next fresh culture dish (PP2) and incubated for another 30 minutes. Again, non-adherent cells and medium were transferred to PP3 and cultured for 1 hour. This was repeated and cultured for additional 22 hours (PP4), 24 hours (PP5) and 72 hours (PP6). The cells adherent to PP6 were defined as MDSCs and treated in the same manner as the BMSCs, described above.[32]

Preparation of Cell-Seeded Collagen Gel Patch

PureCol bovine dermal collagen (2.9 mg/ml, Inamed Corp., Fremont, CA) was prepared following instructions provided by the company. Briefly, 1 mL of sterile, chilled PureCol collagen was mixed with 1.5 mL of sterile 2 × minimal essential medium, 6 µL of sterile 1.75M sodium hydroxide and 0.5 mL distilled water to adjust the pH to 7.4±0.2 and stored on ice to finalize 3 mL of collagen/minimal essential medium solution. Three mL of minimal essential medium supplemented with 20% fetal bovine serum and 2% antibiotics was then added to the iced solution. A 200-µL aliquot of the mixed solution was added to each well of a 48-well dish and incubated at 37°C for 1 hour to prepare the collagen component of the patch.

Confluent plates of BMSCs or MDSCs were washed with phosphate buffered saline solution and then trypsinized as previously described. The cells were centrifuged at 1500 rpm for 5 minutes at room temperature and resuspended in culture media. Final cell density was adjusted to 2.0×10^6 cells/mL. A 100-µL aliquot of the cell suspension was added to each well in the previously incubated collagen gel well.

After incubating at 37°C at 5% CO₂ and 95% air at 100% humidity for 1 day the BMSC or MDSC-seeded gel patch was immediately used for the tendon repair. For the GDF-5 treatment, the gel patch was supplemented with 100 ng/mL rhGDF-5 (Abcam, Cambridge, MA).

Tendon Repair and Tissue Culture

The zone II and zone III tendon regions [33] were used in this study. The zones were separated transversely. Each of the 2 resulting segments was shortened by cutting to a standardized length of 30 mm and then transected centrally. Two simple loop sutures of 6-0 Prolene (Ethicon, Somerville, NJ) were placed at the laceration site. Before tightening the suture loop, 2 identical gel patches were placed between the lacerated tendon ends; afterwards the tendon repair was closed by knotting the suture.

The repaired tendons were placed between 2 wire meshes with longitudinal grooves designed to maintain the tendons in a straight position without applying any tensional force on the tendon (Fig. 1). The meshes with the repaired tendons were cultured in a 100-mm Petri dish with standard culture medium as described above and incubated at 37°C at 5% CO₂ for either 2 or 4 weeks. Culture medium was changed every 3 days. Repaired tendons from zone II were assessed biomechanically. Repaired tendons from zone III were assigned to histological study.

Biomechanical Testing

After the culture period, repaired tendons (n=8) were removed from the culture dish. A single loop suture was placed at opposing ends of the test specimen to connect the tendon to a custom-designed microtester to characterize mechanical properties. The testing apparatus included a load transducer (GSO-50, Transducer Techniques Inc., Temecula, CA), a stepper-motor driven stage, and a potentiometer (TR-50, Novotechnik, Southborough, MA) connected to a hook to catch 1 suture loop and a second hook to connect the other suture loop at the opposite end of a bath/platform. Each test specimen was 40 mm long, which included 5 mm of suture loop at each end and 30 mm of tendon. Before testing, tendon repair sutures were cut without disrupting the repair site, which enabled assessment of the strength of the healing tissue rather than the composite strength of suture and healing.

The tendon rested on a flat, plastic platform moistened with saline. Specimens were distracted at a rate of 0.1 mm/second until the repair site was completely separated. Displacement and failure strength were measured by the transducer and recorded at a sample rate of 100 Hz. Stiffness was defined by the slope linear region of the force/displacement curve.

Histology

To evaluate stem cell migration, the BMSCs and MDSCs were labeled with Vybrant[®] DiI cell labeling solution (Molecular Probes, Eugene, OR) according to the manufacturer's instruction before seeding into the gel patch. After culture, the tendon segment was embedded in optimal cutting temperature compound (Tissue-Tek, Sakura Finetek, Japan) and cut at 7 µm slices with a cryostat (Leica CM 1850, Wetzlar, Germany). These samples were counterstained with 4', 6-diamidino-2-phenylindole (Vector Laboratories, Burlingame, CA) for nuclear staining and observed with a laser scanning confocal microscope (LSM510, Zeiss, Germany). Sections were also stained with hematoxylin and eosin to evaluate the morphology with light microscopy.

Statistical Analysis

The results of the failure strength, stiffness, and gene expression were analyzed by 1-way analysis of variance. The Tukey-Kramer *post hoc* test for each pairwise comparison was performed if there was a significant difference. All results were expressed as means and standard deviations. The significance level was set to $P<0.05$ in all cases.

RESULTS

The failure strength of the repaired tendons after 2 weeks of culture in the MDSC+GDF-5 group was significantly higher than that of the repaired tendons in the no cell group ($P < .001$), the BMSC group ($P < .001$), and the BMSC+GDF-5 group ($P = .019$). After 4 weeks in tissue culture, the failure strength of the repaired tendons in the MDSC+GDF-5 group remained significantly higher than that of the repaired tendons in the no cell group ($P < 0.001$), BMSC group ($P < 0.001$), or MDSC group ($P < 0.001$) (Fig. 2).

The stiffness of the repaired tendons in the MDSC+GDF-5 group was significantly higher than that of the repaired tendons in the no cell group at both 2 and 4 weeks ($P < 0.001$) (Fig. 3).

Qualitatively, hematoxylin and eosin staining revealed that implanted cells were still present between the cut tendon ends at 4 weeks (Fig. 4). Staining with Vybrant[®] DiI indicated that stem cells migrated to the site of injury and repair in all treatments (Fig. 5).

DISCUSSION

After tendon repair, early mobilization is encouraged to prevent adhesions by migration of extrinsic cells that are also responsible for healing.[34] However, early mobilization may also cause gap formation or rupture of the tendon repair. Most ruptures occur around the tenth day.[35] One strategy to reduce the rupture rate, therefore, would be to accelerate and strengthen early tendon intrinsic healing.[36–38] This study investigated the potential of BMSC's and MDSCs combined with GDF-5 to enhance flexor tendon healing. For this purpose, we directly interposed the cells in the tendon laceration site.

The BMSCs have been used in preclinical studies to enhance the healing after tendon repair. Yao et al. showed BMSCs delivered on sutures improved repair strength 7 and 10 days after treatment in a rat Achilles tendon injury model.[39] Interpositioning a BMSC seeded collagen patch between lacerated tendon ends also increased tendon healing strength in a canine *ex vivo* model.[16]

The MDSCs are able to differentiate into several cell lineages and have, like all stem cells, low immunogenicity.[40] Recently, stem-cell-based therapy has been described using MDSCs to treat heart disease,[41] Duchenne muscular dystrophy [42], fractures,[43] and erectile dysfunction [44] in animal models. Yoshimura et al. reported that MDSCs have a higher proliferation potential than BMSCs in a rat model.[45] Considering both the effect of MDSCs in other tissues and the potential for local harvest and given the anatomical continuity of muscle with tendon, we chose MDSCs as a stem cell source for tendon repair.

Sassoon et al. investigated the comparative ability of BMSCs and MDSCs to differentiate into a tenogenic lineage in a cell culture model.[46] In this work, MDSCs demonstrated an increased expression of tenomodulin, a tenocyte marker which plays an important role in tendon development, at 14 days as compared to the BMSCs.

Many cytokines have been studied to enhance tendon healing. Of these, GDF-5 enhances tendon healing. It is “a member of the transforming growth factor- β superfamily”[47] and is also referred to as BMP14 and cartilage-derived morphogenetic protein 1. It has been shown to play a role in tendon development and healing with influences on cell proliferation and differentiation and angiogenesis.[48] Hayashi et al. found that the expression of tenomodulin mRNA increased in BMSCs treated with GDF-5.[18] Park et al. reported that GDF-5 increased adipose-derived mesenchymal stem cell proliferation and enhanced extracellular matrix and tenogenic marker gene expression.[49]

There are various techniques to deliver growth factors, such as injection,[50] viral vectors,[51] collagen sponge,[29] and coated suture.[30, 52] We chose a collagen gel matrix because the gel is easy to manipulate, can be mixed with cells and cytokines during gelation, and thus may be a good means to deliver cells and growth factor directly into the laceration site.

Implantation of MDSCs in combination with GDF-5 accelerated tendon healing at both 2 and 4 weeks. Although the healing strength of the tendon itself was quite low compared to the force measured in *in vivo* tendon repair studies,[53, 54] this is the strength of the healing tissue alone without any suture to supplement the strength. The healing strength of MDSCs with GDF-5 group was about twice as high as the strength of the repaired tendons without added cells or with BMSCs alone at 2 and 4 weeks. We believe that this improvement of *in vitro* intrinsic tendon healing would be amplified *in vivo*, in which many other cytokines, in addition to GDF-5, are present and where extrinsic healing also occurs.

In an *in vitro* lacerated flexor tendon tissue culture environment, the epitenon layer of cells proliferates and migrates into the laceration site.[55] In this study, epitenon cells proliferated and bridged the laceration site in each group.

Furthermore, stem cells remained in and around the repair site for the duration of the study with clear evidence that seeded cells migrated into the injured tendon by 4 weeks.

There are several limitations to this *in vitro* study. Some *in vivo* factors cannot be studied *in vitro*, such as the inflammatory response to injury, the effect of blood supply, and the effect of cytokines other than GDF-5. Second, we did not verify ‘stemness’ of our cells through the presence of surface marker expression, such as CD44, CD73, CD90 or CD105.[56] Third, while we quantified gene expression of both collagen and tenocyte differentiation markers, we did not quantify the resultant gene products by polymerase chain reaction or other measures. Lastly, harvesting MDSCs from normal skeletal muscle may seem too invasive. However, to harvest sufficient cells (1.0×10^7 cells) would require only 10 mg of tissue, making this procedure less invasive than bone marrow aspiration.

Interposition of a GDF-5 treated MDSC-seeded collagen gel patch at the repair site significantly increased tendon healing strength compared to the repaired tendon treated with BMSC-seeded collagen gel patch in an *in vitro* tissue culture model. Further study will be required to investigate this effect *in vivo*.

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Figure 1.

Picture and cartoon of experimental set-up and testing apparatus. (A) Repaired tendons were kept straight, without tension, between the grooves of custom made wire meshes. (B) The loops stitched on the end of tendon were hitched to the hooks. Both sutures were cut at both sides before testing.

Figure 2.

Mean failure strength of the repaired tendon. Error bars represent standard deviation. An asterisk indicates a significant difference ($P < 0.05$).

Figure 3. Mean stiffness of the repaired tendon. Error bars represent standard deviation. An asterisk indicates a significant difference ($P < 0.05$).

Figure 4.

Hematoxylin and eosin staining of healing tendons in treatment groups at 4 weeks. (Original magnification x100. Scale bar represents 100 μ m). Epitenon cells bridge the repaired site in each group in all treatments; No cell group (A), BMSC group (B), MDSC group (C), BMSC +GDF-5 group (D) and MDSC+GDF-5 group (E).

Figure 5.

Bone marrow stromal cells and muscle mesenchymal stem cells stained with Vybrant[®] DiI cell labeling solution (Molecular Probes, Eugene, OR) (red) and counter stained with 4', 6-diamidino-2-phenylindole (Vector Laboratories, Burlingame, CA) (blue) were observed under confocal microscopy 4 weeks. (Original magnification x100. Scale bar represents 100μm). (A) no cell group, (B) BMSC group, (C) MDSC group, (D) BMSC+GDF-5 group and (E) MDSC+GDF-5 group. Note migration of red stained cells into the adjacent tendon, especially in the 2 MDSC specimens (5C and 5E).

Table 1

Experimental Design and Sample Number

Groups	Description	Culture 2 week		Culture 4 week	
		Biomechanics	Histology	Biomechanics	Histology
		Zone II	Zone III	Zone II	Zone III
1	No cell	8	3	8	3
2	BMSC	8	3	8	3
3	MDSC	8	3	8	3
4	BMSC+GDF-5	8	3	8	3
5	MDSC+GDF-5	8	3	8	3