

Mesenchymal Stem Cells and Insulin-Like Growth Factor-I Gene-Enhanced Mesenchymal Stem Cells Improve Structural Aspects of Healing in Equine Flexor Digitorum Superficialis Tendons

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ABSTRACT: Tendinitis remains a catastrophic injury among athletes. Mesenchymal stem cells (MSCs) have recently been investigated for use in the treatment of tendinitis. Previous work has demonstrated the value of insulin-like growth factor-I (IGF-I) to stimulate cellular proliferation and tendon fiber deposition in the core lesion of tendinitis. This study examined the effects of MSCs, as well as IGF-I gene-enhanced MSCs (AdIGF-MSCs) on tendon healing in vivo. Collagenase-induced bilateral tendinitis lesions were created in equine flexor digitorum superficialis tendons (SDFT). Tendons were treated with 10×10^6 MSCs or 10×10^6 AdIGF-MSCs. Control limbs were injected with 1 mL of phosphate-buffered saline (PBS). Ultrasound examinations were performed at $t = 0, 2, 4, 6,$ and 8 weeks. Horses were euthanized at 8 weeks and SDFTs were mechanically tested to failure and evaluated for biochemical composition and histologic characteristics. Expression of collagen types I and III, IGF-I, cartilage oligomeric matrix protein (COMP), matrix metalloproteinase-3 (MMP-3), matrix metalloproteinase-13 (MMP-13), and aggrecanase-1 (ADAMTS-4) were similar in MSC and control tendons. Both MSC and AdIGF-MSC injection resulted in significantly improved tendon histological scores. These findings indicate a benefit to the use of MSCs and AdIGF-MSCs for the treatment of tendinitis. © 2009 Orthopaedic Research Society. Published by Wiley Periodicals, Inc. *J Orthop Res*

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Tendinitis remains a catastrophic injury amongst athletes. Tendon injuries are predominantly degenerative in nature and are often recalcitrant to treatment, being slow to heal, and rarely regaining their original strength and elasticity. Despite improvements in the early detection and serial evaluation of damaged tendons using ultrasound, and advances in rehabilitation techniques and treatments, a consistently successful treatment regimen has yet to be developed.

Recent work has focused on the use of mesenchymal stem cells (MSCs) as well as growth factors to improve the quality and speed of healing in tendinitis repair. MSCs are the pluripotent precursor cells of connective tissues and are believed to play an important role in tendon and ligament repair, fracture healing, and cartilage resurfacing.^{1–5} Although MSCs have been used empirically for the treatment of tendinitis in larger species for the past several years,^{6,7} there are very few controlled studies documenting the efficacy of MSCs for tendinitis lesion repair in large animal models.⁸

A combination of MSCs and anabolic growth factors would seem very useful as an enhanced cell therapy repair technique. Growth factors are peptide signaling molecules that regulate many aspects of cellular metabolism including the cell cycle, cell growth and differentiation, and the production and destruction of extracellular matrix products.^{9,10} Their effects are mediated primarily via autocrine and paracrine mechanisms, which provides the rationale for local administration of exogenous growth factors to influence cellular metabolism.¹¹ Previous work has demonstrated the

value of growth factor injections, particularly insulin-like growth factor-I (IGF-I), to stimulate cellular proliferation and matrix synthesis in animal tendon and ligament models.^{12–20} Although exogenous IGF-I has been shown to stimulate tendon healing in vivo in an equine model,²⁰ it has a short half-life, which necessitates repeated dosing, making clinical application challenging and costly. These problems could be abolished by a gene therapy approach to insert the IGF-I gene directly into healing tendon lesions. The objective of this study, therefore, was to examine the effects of MSCs as well as IGF-I gene-enhanced MSCs (AdIGF-MSCs) on tendon healing in vivo using an equine collagenase model of flexor tendinitis.

MATERIALS AND METHODS

Study Design

For these studies, 12 young adult horses, ranging in age from 2–5 years (five male, seven female) were used. All horses were examined clinically and ultrasonographically to rule out preexisting tendinitis, and all horses were treated with their own cultured stem cells. This project was approved and performed according to guidelines of the Institutional Animal Care and Use Committee of Cornell University.

Sternal bone marrow aspirates were obtained by using a Jamshidi bone marrow biopsy needle under sterile conditions following local anesthesia. A total of 120 mL of bone marrow aspirate was collected from each horse into two 60-mL syringes containing heparin. MSCs were then grown to confluency and stored frozen as previously described.²¹ Briefly, cell isolation consisted of plating marrow aspirate in low glucose Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum and 0.0125 $\mu\text{g/mL}$ of bFGF. The cultures were then left undisturbed for 3 days to allow the cells to attach to the culture plasticware. After the initial 3 days, the medium was replaced every other day. To expand cell numbers, confluent monolayers

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of cells were trypsinized and passaged as needed. All cultures were maintained at 5% CO₂, 90% humidity, and 37°C. When the passaged cells had reached confluency, they were trypsinized, resuspended in freeze media (medium with 10% fetal bovine serum and 10% dimethyl sulfoxide), and frozen at 5×10^6 cells/vial until used for injection.

Collagenase-induced lesions were created in the tensile region of the superficial digital flexor tendons (SDFT) of both forelimbs using filter sterilized bacterial collagenase type I (Sigma, St. Louis, MO) diluted in sterile water. A total of 2000 units of collagenase were delivered to two sites centered in the tensile region of the SDFT tendon under ultrasonographic guidance, as described by this group previously.^{20,22} The horses were confined to box stalls and their forelimbs bandaged. Each horse served as its own control, with one forelimb randomly assigned as the treated limb and the other as the control. Five days postcollagenase injection ($t = 0$), six of the horses were treated with 10×10^6 MSCs and six were treated with 10×10^6 AdIGF-MSCs suspended in 1 mL of phosphate-buffered saline (PBS). Control limbs were injected with 1 mL of PBS. All injections were performed under ultrasonographic guidance to confirm injection into the lesion. Ten days prior to injection, frozen cell aliquots were thawed and cultured. AdIGF-MSCs were transduced with 500 MOI of AdIGF-I overnight in preparation for injection the following day.²³ Briefly, medium was removed from two culture flasks each containing approximately 10×10^6 cells from each horse in the AdIGF-MSC group, the cell layer rinsed with Hanks solution, and adenoviral inoculum added to each flask using a dose based on actual cell number per flask to achieve 500 MOI. The flasks were incubated with the virus for 1.5 h with gentle rocking every 15 min. The medium was then changed to high glucose Dulbecco's modified Eagle's medium supplemented with 1% ITS+, 50 µg/mL of ascorbic acid, 100 µg/mL of sodium pyruvate, and 40 µg/mL of proline and incubated overnight. Real-time polymerase chain reaction (PCR) was used to confirm IGF-I gene expression. IGF-I gene expression in AdIGF-MSCs was significantly greater than in naïve MSCs ($n = 6$; $p \leq 0.001$; paired t -test). IGF-I gene expression (copy#/µg RNA) was an average 160,000-fold greater in AdIGF-MSCs ($168,109 \pm 59,624$ copy#/µgRNA) than in naïve MSCs (1.5 ± 0.17 copy#/µgRNA).

Horses were confined to box stalls for the duration of the 8-week study and their forelimbs bandaged. Ultrasound examinations of the tendons were performed at $t = 0, 2, 4, 6,$ and 8 weeks. Cross-sectional area of the tendon and lesion, echogenicity, loss of linear fiber pattern, and compressibility were measured by the same blinded ultrasonographer (A.E.Y.).

Tissue Harvest and Mechanical Testing

Horses were euthanized at 8 weeks posttreatment and the entire SDFTs were harvested for analysis under RNase-free conditions and stored on ice for immediate mechanical testing. Tendons were loaded at a rate of 40 mm/s to failure in a servohydraulic testing system (Bionix, MTS Systems Corporation, Eden Prairie, MN) using custom-designed cryoclamps. Load and displacement data were collected digitally at 10 Hz. Midsubstance strain was recorded by videotape using markers fixed to the surface of the tendon. Load data were normalized by CSA to determine stress, and modulus was calculated from the slope of the linear portion of the stress/strain curve. Following mechanical testing, entire tendon longitudinal segments through the healing lesion were collected. Tissue samples were collected from the grossly damaged areas,

extending into the surrounding normal tendon. Tendon samples were snap-frozen in liquid nitrogen for gene expression studies, rinsed in protease inhibitors in water and snap frozen in liquid nitrogen for biochemical analysis, or fixed in 4% paraformaldehyde at 4°C for histology. Tendon samples for molecular and biochemical analyses were then pulverized in a freezer-mill and stored at -80°C until use.

RNA Isolation and Quantitative RT/PCR

Total cellular RNA was isolated using Trizol (Invitrogen, Carlsbad, CA) according to the manufacturer's directions with minor modifications as previously described,²⁴ and precipitated RNA was further purified using RNeasy spin columns (Qiagen, Chatsworth, CA). RNA was assessed by spectrophotometry at 260:280 nm and by 1% agarose gel electrophoresis. RNA was used for quantitative RT/PCR of collagen types I and III,^{22,24} IGF-I,²⁵ and cartilage oligomeric matrix protein (COMP),^{26–28} as indicators of tendon matrix synthesis, and matrix metalloproteinase-3 (MMP-3),^{29–31} matrix metalloproteinase-13 (MMP-13),^{24,29,32} and aggrecanase (ADAMTS-4)^{30,33} as indicators of tendon catabolism.

Total RNA was reverse transcribed and amplified using the One-Step RT-PCR technique and the ABI PRISM 7900 Sequence Detection System (Applied Biosystems, Foster City, CA). The primers and dual-labeled fluorescent probe (6-FAM as the 5' label (reporter dye) and TAMRA as the 3' label (quenching dye) were designed using Primer Express Software version 2.0b8a (Applied Biosystems). All probes and primers were designed using equine specific sequences either published in Genbank, or sequenced in our laboratory and described previously.^{34,35} Total copy number of each gene was obtained from a standard curve and normalized to 18s RNA expression.

Biochemical Analysis

Pulverized tendon samples were lyophilized and digested in papain (1 mL papain/10 mg lyophilized tendon) at 65°C. Total DNA content was determined in duplicate aliquots of tendon samples digested in papain for 24 h. The samples were mixed with bisbenzimidazole compound for DNA quantification by fluorometric assay.³⁶ Total glycosaminoglycan content was determined in duplicate aliquots of samples digested in papain for 4 h. The samples were mixed with dimethylmethylene blue dye for glycosaminoglycan quantification by colorimetric assay.³⁷ Total soluble collagen content was determined in lyophilized tendon samples using the Sircol Assay (Biocolor Ltd., Newtownabbey, Northern Ireland) according to the manufacturer's directions for pepsin soluble collagens with modifications as previously described.²⁴

Histology

Paraformaldehyde-fixed longitudinal tissue sections for histology were dehydrated, cleared in xylene, embedded in paraffin, sectioned, mounted on microscope slides, and stained with hematoxylin and eosin (H&E). Sections for collagen type I immunohistochemistry were deparaffinized, rehydrated, and treated with 5 µL/mL hyaluronidase (Sigma-Aldrich, St. Louis, MO) at 37°C for 60 min to facilitate antibody penetration into the tissue. After blocking with nonimmune goat serum, sections were incubated with polyclonal rabbit anti-equine type I collagen primary antibody diluted 1:100 in PBS for 60 min at 37°C. The sections were washed with PBS and the secondary goat antirabbit antibody labeled with Alexa fluorescent 488 (Invitrogen, Carlsbad, CA) was applied. Cell

nuclei were counterstained with propidium iodide, and examined under confocal fluorescent microscopy to determine type I collagen distribution. Negative procedural control samples consisting of tendon sections incubated with non-immune goat serum instead of primary antibody were included on each slide. All slides were examined by two blinded observers (A.J.N. and L.V.S.), using a calibrated reticule to sequentially examine across and down the entire tendon section, using low power and high power where appropriate for cell detail, to derive a complete histologic impression. Scores were assigned after examining at least two independent sections from each tendon. All tendon parameters were scored from 1 (normal) to 4 (severe changes) for: tenocyte shape, tenocyte density, free hemorrhage, neovascularization, perivascular cuffing, collagen fiber linearity, collagen fiber uniformity, polarized light crimping, and collagen type I deposition as previously described.²² Scores from both observers were averaged. This grading scheme expands on previously described systems which utilize a four-parameter score.^{38,39}

Statistical Analysis

For statistical purposes, each animal served as its own control. Numerical data were analyzed using paired *t*-tests when comparing treated tendons to their respective controls and two-sample *t*-tests when comparing treated tendons to each other. Ordinal data (scores) were analyzed using Wilcoxon signed rank tests when comparing treated tendons to their respective controls or Wilcoxon rank sum tests when comparing treated tendons to each other. When comparing treated tendons to each other as well as their controls, a one-way analysis of variance (ANOVA) and Least Significant Difference (LSD) post hoc test was used. For all tests, Statistix 8 software (Analytical Software, Tallahassee, FL) was used and significance was set at $p < 0.05$.

RESULTS

Ultrasound Data

There were no significant differences in ultrasound parameters between treated tendons and their respective controls or between MSC and AdIGF-MSC-treated tendons (data not shown). All parameters were normalized as a percent of the baseline measurement or score of the lesion on the first day of treatment. As expected, the lesion CSA, grade of echogenicity, and lesion compressibility all increased compared to their baseline measurements during the first 2 to 4 weeks and then gradually decreased, while the percentage increase in linear fiber pattern compared to baseline was initially negative and then gradually increased in all groups.

Mechanical Testing

By the time mechanical testing was performed at 8 weeks posttreatment, the tendon lesions had healed well enough to withstand sizeable tensile loads and only 1 of the 24 tendons failed at the lesion. Of the remaining tendons, 21 failed proximal and 2 failed distal to the lesion. As ultimate failure loads were limited by the clamps used and extent of normal tissue present, the most critical parameter was the tensile modulus. Both MSC and AdIGF-MSC-treated tendons were stiffer than their respective controls; however, these results

were not significant (paired *t*-test; $p = 0.46$ and $p = 0.20$, respectively). AdIGF-MSC-treated tendons were stiffer than MSC-treated tendons; however, this difference was not statistically significant (two sample *t*-test; $p = 0.10$) (Fig. 1).

Gene Expression

There were no significant differences in anabolic (collagen type I and 3, COMP, or IGF-I) gene expression between MSC and AdIGF-MSC-treated tendons or between treated and control tendons within the AdIGF-MSC group. In the MSC group, COMP expression in control tendons was greater than COMP expression in MSC treated tendons (Table 1). There were no significant differences in catabolic (ADAMTS-4, MMP-13, or MMP-3) gene expression between treated tendons and their respective controls within both the MSC and AdIGF-MSC groups. AdIGF-MSC-treated tendons, however, had increased MMP-13 gene expression compared to MSC-treated tendons (Table 1).

Biochemical Analysis

There were no significant differences in DNA, glycosaminoglycan, or total collagen content between treated tendons and their respective controls or between MSC and AdIGF-MSC-treated tendons (Table 2).

Histology

Cumulative histology scores were significantly lower (more normal) for both MSC and AdIGF-MSC-treated tendons compared to their respective controls (Table 3 and Fig. 2). Several individual parameters, including collagen type I immunohistochemistry, were significantly more normal in AdIGF-MSC-treated tendons compared to their controls, whereas in the MSC group no individual parameters were significantly different between treated and control tendons (Table 2 and Figs. 2 and 3). The cumulative score for AdIGF-MSC-treated tendons was also lower (more normal than MSC-

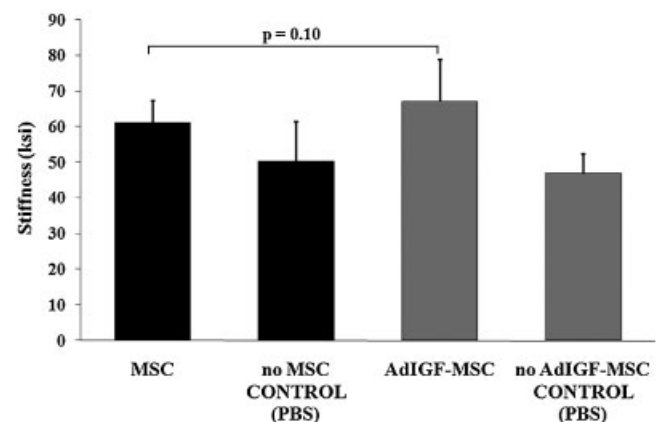


Figure 1. Mean tensile modulus (ksi) of treated and control tendons within both MSC and AdIGF MSC groups. Bars represent mean ($n = 6$) \pm SE. Both MSC and AdIGF-MSC-treated tendons were stiffer than their respective controls; however, these results were not significant. In addition, AdIGF-MSC-treated tendons were stiffer than MSC-treated tendons, but these results were also not significant (two sample *t*-test; $p = 0.10$).

Table 1. Quantitative PCR Analysis of Gene Expression in Treated and Control Tendons

Gene Expression (Copy No./ μg RNA):18s RNA	MSC	No MSC Control (PBS)	AdIGF-MSC	No AdIGF-MSC Control (PBS)
Col I	$5.68 \times 10^5 \pm 0.70$	$6.70 \times 10^5 \pm 0.77$	$4.91 \times 10^5 \pm 0.62$	$6.65 \times 10^5 \pm 0.93$
Col III	$3.20 \times 10^6 \pm 0.45$	$3.52 \times 10^6 \pm 0.34$	$2.41 \times 10^6 \pm 0.30$	$3.45 \times 10^6 \pm 0.38$
COMP	$0.93 \times 10^5 \pm 0.21$	$1.12 \times 10^5 \pm 0.17^a$	$1.17 \times 10^5 \pm 0.17$	$0.89 \times 10^5 \pm 0.23$
IGF-I	$1.77 \times 10^3 \pm 0.28$	$1.63 \times 10^3 \pm 0.26$	$1.57 \times 10^3 \pm 0.18$	$1.72 \times 10^3 \pm 0.37$
ADAMTS-4	$3.48 \times 10^2 \pm 1.06$	$2.00 \times 10^2 \pm 0.28$	$3.11 \times 10^2 \pm 0.49$	$3.31 \times 10^2 \pm 0.54$
MMP-13	$1.91 \times 10^2 \pm 0.30$	$2.81 \times 10^2 \pm 0.77$	$3.07 \times 10^2 \pm 0.99^b$	$4.30 \times 10^2 \pm 1.10$
MMP-3	$0.22 \times 10^2 \pm 0.09$	$0.18 \times 10^2 \pm 0.05$	$0.23 \times 10^2 \pm 0.08$	$0.18 \times 10^2 \pm 0.05$

Data are presented as the mean ($n = 6$) \pm SE. ^aIndicates significant differences between treated tendons and their respective controls (paired t -test; $p < 0.05$). ^bIndicates significant differences between MSC and AdIGF-MSC-treated tendons (two-sample t -test; $p < 0.05$).

treated tendons); however, the difference was not significant (Wilcoxon rank sum test; $p = 0.62$).

DISCUSSION

The aims of this study were to evaluate the efficacy of MSCs for the treatment of tendinitis and to determine whether a gene therapy approach with MSCs could be used to exploit the beneficial effects of IGF-I on tendon healing in vivo. Both MSC and Ad IGF-MSC injections into tendinitis core lesions resulted in significant histological tendon healing and a trend towards improved biomechanical characteristics of healing tendon in the early period after injury. Few differences, however, were found between MSC and AdIGF-MSC-treated tendons, suggesting that further work is needed to enhance IGF-I gene delivery.

Much of the recent literature surrounding the use of MSCs in larger species has focused on clinical experience alone and is hindered by small case numbers without significant follow-up times and without controls.^{6,7,40} The collagenase-induced model of tendinitis is well documented and has been used extensively in equine and lab animal studies as a model for human tendinitis.^{20,22,25,41–45} The use of this technique to create a suitable model for the study of naturally occurring tendinitis has been supported by the evaluation of gross and histopathological changes, clinical signs, and ultrasonographic findings following the induction of injury.^{44,46–48} In addition, our laboratory has successfully investigated the effects of recombinant IGF-I on equine tendon healing using this collagenase induced model of tendinitis.²⁰

The histologic, biochemical, and mechanical characteristics of the injured tendons in this study are consistent with those described in previous reports using

the collagenase induced model.^{20,44,46,47,49} All tendons had anechoic core lesions and an increase in CSA on ultrasound examination following collagenase injection indicative of acute matrix destruction. Interestingly, however, no differences in ultrasonographic parameters between MSC and AdIGF-MSC-treated tendons and their respective controls were found during the 8-week healing period posttreatment despite significant improvement in histologic evaluation in the MSC and AdIGF-MSC-treated tendons at the termination of the study. The insensitivity of ultrasound to provide fine structural detail has been described previously in adipose-derived stem cells injected into this same model.²²

When mechanically tested to failure at 8 weeks posttreatment, all tendons had healed well enough to withstand high tensile loads, and only 1 of 24 (4%) of the tendons failed at the lesion. As ultimate failure loads are limited by the clamps used and the extent of normal tissue present, lack of failure at the lesion site is a commonly reported finding,²⁰ and tensile modulus measurements, rather than ultimate failure loads, are used as the critical assessment of mechanical tendon healing. Both MSC and AdIGF-MSC-treated tendons showed a trend toward increased modulus compared to their respective PBS-treated controls. In addition, AdIGF-MSC-treated tendons showed a trend toward increased tensile modulus compared to MSC-treated tendons. These results are consistent with the histologic findings in this study and with the previously reported IGF-I in vivo tendon healing study, in which treatment of collagenase-induced tendon lesions with recombinant IGF-I resulted in a trend toward increased modulus compared to untreated controls.²⁰

The DNA content of the lesion tissue was similar in all tendons, regardless of previous MSC cell injection. This

Table 2. Biochemical Analysis of DNA, Glycosaminoglycan, and Total Collagen Content of Treated and Control Tendons

Molecule	MSC	No MSC Control (PBS)	AdIGF-MSC	No AdIGF-MSC Control (PBS)
DNA (ng/ μg)	1.55 ± 0.35	1.85 ± 0.45	1.62 ± 0.17	1.38 ± 0.23
Glycosaminoglycan ($\mu\text{g}/\text{mg}$)	12.79 ± 1.35	10.70 ± 2.24	10.26 ± 1.56	7.96 ± 1.19
Total Collagen ($\mu\text{g}/\text{mg}$)	330.96 ± 20.75	298.67 ± 17.75	245.85 ± 27.89	308.75 ± 26.73

Data are presented as the mean ($n = 6$) \pm SE.

Table 3. Histological Scores for Treated and Control Tendons

Tendon Parameter	MSC	No MSC Control (PBS)	AdIGF-MSC	No AdIGF-MSC Control (PBS)
Cell shape	2.50 ± 0.22	2.75 ± 0.17	2.46 ± 0.25	2.67 ± 0.21
Cell density	2.50 ± 0.22	3.00 ± 0.00	2.13 ± 0.18**	3.08 ± 0.20
Free hemorrhage	1.58 ± 0.33	2.25 ± 0.17	1.33 ± 0.25	1.67 ± 0.25
Neovascularization	2.33 ± 0.21	2.75 ± 0.17	1.92 ± 0.08	2.42 ± 0.15
Perivascular cuffing	1.58 ± 0.30	2.08 ± 0.08	1.50 ± 0.18	1.83 ± 0.17
Collagen linearity	2.00 ± 0.00	2.50 ± 0.22	1.83 ± 0.31**	3.17 ± 0.17
Collagen uniformity	2.00 ± 0.00	2.67 ± 0.21	2.08 ± 0.33*	3.17 ± 0.17
Polarized crimping	2.33 ± 0.33	3.00 ± 0.00	2.00 ± 0.45*	3.50 ± 0.22
Epitenon thickening	2.50 ± 0.22	3.00 ± 0.00	2.33 ± 0.21	2.67 ± 0.21
Col I Immunohistochemistry	2.58 ± 0.15	3.17 ± 0.31	2.50 ± 0.22*	3.25 ± 0.21
Cumulative score	21.92 ± 1.66** ^a	27.17 ± 0.46 ^b	19.92 ± 1.84** ^a	27.42 ± 0.86 ^b

Each parameter scored from 1 (normal) to 4 (most damaged); the resultant cumulative score is from 10 (most normal) to 40 (most damaged). Data are presented as the mean (n = 6) ± SE. *Indicates significant differences between treated tendons and their respective controls (Wilcoxon signed rank test; **p < 0.05; *p < 0.06). Superscript letters indicate significant differences between treatment groups when all groups were compared together (p < 0.05).

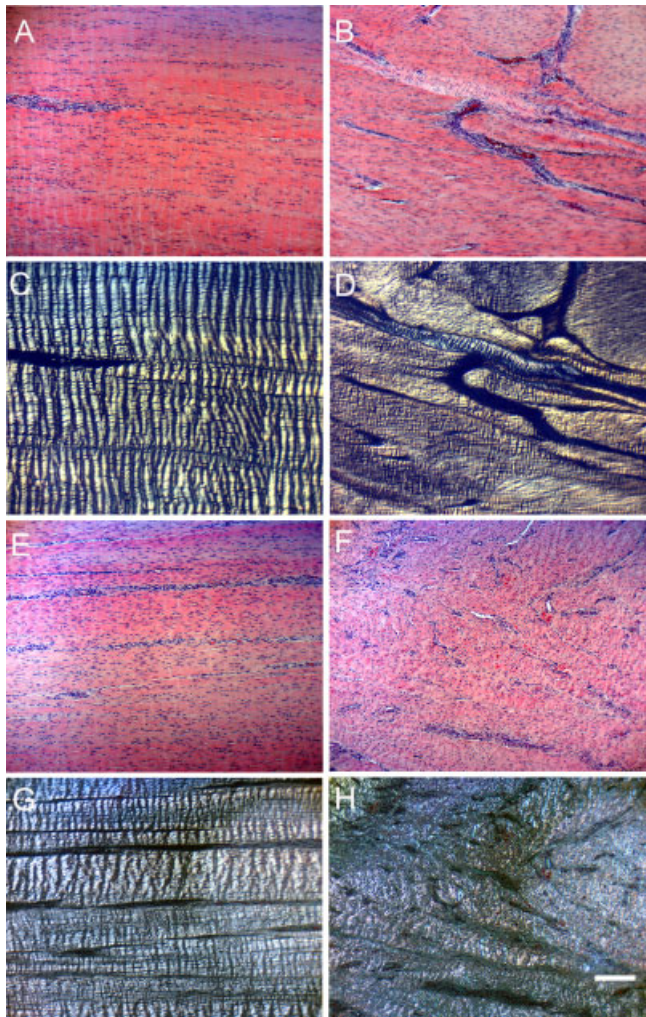


Figure 2. H&E stained longitudinal sections of (a) MSC-treated and (b) control tendons, and (e) AdIGF-MSC-treated and (f) control tendons. Collagen fiber crimping for (c) MSC-treated and (d) control tendons, and (g) AdIGF-MSC-treated and (h) control tendons examined under polarized light. Bar = 200 µm.

suggests that many of the injected MSCs did not persist or potentially reduced cohort inflammatory cell influx. Several histologic parameters including cell density, regions of hemorrhage, blood vessel abundance, perivascular cuffing, and epitenon thickening all trended lower in MSC and AdIGF-MSC-treated tendons. Such a visible anti-inflammatory effect, while beneficial in many regards, also drives down DNA content, and therefore masks any MSC persistence data. It is clear that injecting genetically or membrane dye labeled cells would be ideal to gauge cell survival in follow-up studies.⁵⁰

Similar DNA and total collagen content in AdIGF-MSC-treated tendons and their controls contrasts to previous IGF-I tendon healing studies,^{12,14,15,20,51,52} and

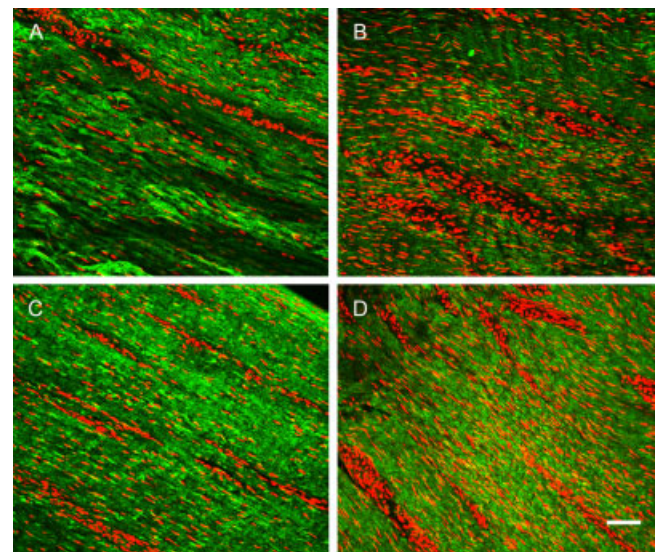


Figure 3. Collagen type I abundance shown by confocal immunohistochemistry of (a) MSC-treated and (b) control tendons, and (c) AdIGF-MSC-treated and (d) control tendons. Collagen type I is shown by green Alexa 488 reaction and propidium incorporation indicates cell nuclei (red). Bar = 120 µm.

suggests that the IGF-I transgene expression may persist for only a short period in the AdIGF-MSC-treated tendons. This finding is supported by the fact that there was no difference in IGF-I gene expression between AdIGF-MSC-treated tendons and their controls at the 8-week termination of the study, while there was a significant difference when compared to naïve cells immediately after transduction. Similarly, there was no correlation between IGF-I gene expression in AdIGF-I transduced cells and final histologic score for each animal (data not shown). The transduction dose of 500 MOI of AdIGF-I used was previously determined in our laboratory for optimal MSC cell survival and IGF-I gene expression *in vitro*,²³ but may not be the optimal dose *in vivo*. Adenovirus delivery frequently results in only 4 weeks of transgene activity before a combination of gene silencing and carrier cell loss eliminate gene product.^{35,53} In addition, while the adenovirus vector is regarded as only moderately immunogenic, it is likely that neutralizing antibodies may have limited the transgene response and that the use of a less immunogenic vector such as adeno-associated virus (AAV) could improve IGF-I persistence in tendon.

MSC and AdIGF-MSC-treated tendons had significantly more normal cumulative histologic scores compared to their respective controls. This finding, along with the lack of significant differences in biochemical parameters (DNA, glycosaminoglycan, and total collagen content) and gene expression between MSC and AdIGF-MSC-treated tendons and controls, suggests that the predominant effect of MSCs on tendon healing is through tendon structural organization rather than cell numbers or anabolic gene expression. Quantitative parameters accurately reflect constituents of the healing tendon, but do little to measure the organization of tendon that was provided here by histologic scores. A combination of data is clearly more useful than any one measure alone. Additionally, the dose of MSCs used in this study (10×10^6 cells) was based on the authors' clinical experience and clinical reports in the literature,^{7,54} as to date there are no studies that critically evaluate the optimal number of MSCs that should be used in the treatment of tendinitis. It is possible that optimization of a MSC dose in future studies could affect biochemical and gene expression results.

In conclusion, these findings support the use of MSCs for the treatment of tendinitis. Both MSC and AdIGF-MSC intralesional injection resulted in significantly better tendon histological scores and a trend toward improved biomechanical characteristics. The added value of IGF-I gene-enhanced MSC implantation was minimal compared to naïve MSC injection. Further investigation into the optimal dose of MSCs and the potential of different dosing, extended IGF-I expression, or less immunogenic viral vectors for IGF-I delivery, would seem warranted before clinical application.

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REFERENCES

1. Wakitani S, Goto T, Pineda SJ, et al. 1994. Mesenchymal cell-based repair of large, full-thickness defects of articular cartilage. *J Bone Joint Surg* 76-A:579–592.
2. Grande DA, Southerland SS, Manji R, et al. 1995. Repair of articular cartilage defects using mesenchymal stem cells. *Tissue Eng* 1:345–353.
3. Lazarus HM, Haynesworth SE, Gerson SL, et al. 1995. Ex vivo expansion and subsequent infusion of human bone marrow-derived stromal progenitor cells (mesenchymal progenitor cells): implications for therapeutic use. *Bone Marrow Transplant* 16:557–564.
4. Bruder SP, Fink DJ, Caplan AI. 1994. Mesenchymal stem cells in bone development, bone repair, and skeletal regeneration therapy. *J Cell Biochem* 56:282–294.
5. Bruder SP, Jaiswal N, Ricalton NS, et al. 1998. Mesenchymal stem cells in osteobiology and applied bone regeneration. *Clin Orthop* 355S:S247–S256.
6. Smith RK, Korda M, Blunn GW, et al. 2003. Isolation and implantation of autologous equine mesenchymal stem cells from bone marrow into the superficial digital flexor tendon as a potential novel treatment. *Equine Vet J* 35:99–102.
7. Smith RK, Webbon PM. 2005. Harnessing the stem cell for the treatment of tendon injuries: heralding a new dawn? *Br J Sports Med* 39:582–584.
8. Crovace A, Lacitignola L, De SR, et al. 2007. Cell therapy for tendon repair in horses: an experimental study. *Vet Res Commun* 31(Suppl 1):281–283.
9. Bradshaw R, Cavanaugh K. 1990. Isolation and characterization of growth factors. In: Sporn M, Roberts A, editors. *Peptide growth factors and their receptors 1*. 1st ed. New York: Springer-Verlag. p 17–65.
10. Rechler MM, Nissley SP. 1990. Insulin-like growth factors. In: Sporn MB, Roberts AB, editors. *Peptide growth factors and their receptors*. New York: Springer Verlag.
11. Sporn M, Roberts A. 1990. The multifunctional nature of peptide growth factors. In: Sporn M, Roberts A, editors. *Peptide growth factors and their receptors 1*. 1st ed. New York: Springer-Verlag. p 3–15.
12. Abrahamsson S-O, Lundborg G, Lohmander LS. 1991. Long-term explant culture of rabbit flexor tendon: Effects of recombinant human insulin-like growth factor-I and serum on matrix metabolism. *J Orthop Res* 9:503–515.
13. Lee J, Green MH, Amiel D. 1994. The study of growth factors and their synergy on cell outgrowth from rabbit anterior cruciate and medial collateral ligament explants. *Trans 40th Ann Mtg ORS*; 19:60.
14. Tsuzaki M, Brigman BE, Xiao H, et al. 1994. IGF-I and TGF-B drive tendon cell DNA synthesis. *Trans 40th Ann Mtg ORS*; 19:18.
15. Abrahamsson S-O, Lundborg G, Lohmander LS. 1991. Recombinant human insulin-like growth factor-I stimulates *in vitro* matrix synthesis and cell proliferation in rabbit flexor tendon. *J Orthop Res* 9:495–502.
16. Hansson H-A, Dahlin LB, Lundborg G, et al. 1988. Transiently increased insulin-like growth factor I immunoreactivity in tendons after vibration trauma. *Scand J Plast Reconstr Surg Hand Surg* 22:1–6.

17. Banes AJ, Tsuzaki M, Hu P, et al. 1995. PDGF-BB, IGF-I and mechanical load stimulate DNA synthesis in avian tendon fibroblasts in vitro. *J Biomech* 28:1505–1513.
18. DesRosiers EA, Yahia L, Rivard C-H. 1996. Proliferative and matrix synthesis response of canine anterior cruciate ligament fibroblasts submitted to combined growth factors. *J Orthop Res* 14:200–208.
19. Abrahamsson SO, Lohmander S. 1996. Differential effects of insulin-like growth factor-I on matrix and DNA synthesis in various regions and types of rabbit tendons. *J Orthop Res* 14:370–376.
20. Dahlgren LA, van der Meulen MC, Bertram JE, et al. 2002. Insulin-like growth factor-I improves cellular and molecular aspects of healing in a collagenase-induced model of flexor tendinitis. *J Orthop Res* 20:910–919.
21. Wilke MM, Nydam D, Nixon AJ. 2007. Enhanced early chondrogenesis in articular defects following arthroscopic mesenchymal stem cell implantation in an equine model. *J Orthop Res* 25:913–925.
22. Nixon AJ, Dahlgren LA, Haupt JL, et al. 2008. Effect of adipose-derived nucleated cell fractions on tendon repair in horses with collagenase-induced tendinitis. *Am J Vet Res* 69:928–937.
23. Haupt JL, Nixon AJ. 2007. Gene enhanced mesenchymal stem cells for the treatment of tendonitis. *Trans 52nd Ann Mtg ORS*.
24. Schnabel LV, Mohammed HO, Miller BJ, et al. 2007. Platelet rich plasma (PRP) enhances anabolic gene expression patterns in flexor digitorum superficialis tendons. *J Orthop Res* 25:230–240.
25. Dahlgren LA, Mohammed HO, Nixon AJ. 2005. Temporal expression of growth factors and matrix molecules in healing tendon lesions. *J Orthop Res* 23:84–92.
26. Sodersten F, Ekman S, Eloranta ML, et al. 2005. Ultrastructural immunolocalization of cartilage oligomeric matrix protein (COMP) in relation to collagen fibrils in the equine tendon. *Matrix Biol* 24:376–385.
27. Smith RK, Gerard M, Dowling B, et al. 2002. Correlation of cartilage oligomeric matrix protein (COMP) levels in equine tendon with mechanical properties: a proposed role for COMP in determining function-specific mechanical characteristics of locomotor tendons. *Equine Vet J Suppl* 241–244.
28. Smith RKW, Zunino L, Webbon PM, et al. 1997. The distribution of cartilage oligomeric matrix protein (COMP) in tendon and its variation with tendon site, age and load. *Matrix Biol* 16:255.
29. Oshiro W, Lou J, Xing X, et al. 2003. Flexor tendon healing in the rat: a histologic and gene expression study. *J Hand Surg Am* 28:814–823.
30. Jones GC, Corps AN, Pennington CJ, et al. 2006. Expression profiling of metalloproteinases and tissue inhibitors of metalloproteinases in normal and degenerate human achilles tendon. *Arthritis Rheum* 54:832–842.
31. Alfredson H, Lorentzon M, Backman S, et al. 2003. cDNA-arrays and real-time quantitative PCR techniques in the investigation of chronic Achilles tendinosis. *J Orthop Res* 21:970–975.
32. Fortier LA, Schnabel LV, Mohammed HO, et al. 2007. Assessment of cartilage degradation effects of matrix metalloproteinase-13 in equine cartilage cocultured with synovio-cytes. *Am J Vet Res* 68:379–384.
33. Goodrich LR, Chen CT, Hidaka C, et al. 2007. Catabolic cytokine profiles of cartilage adjacent to chondral defects explain the biomechanically inferior tissue. *Trans 52nd Ann Mtg ORS* 31:4–6.
34. Dahlgren LA, Brower-Toland BD, Nixon AJ. 2005. Cloning and expression of type III collagen in normal and injured tendons of horses. *Am J Vet Res* 66:266–270.
35. Goodrich LR, Hidaka C, Robbins PD, et al. 2007. Genetic modification of chondrocytes with insulin-like growth factor-1 enhances cartilage healing in an equine model. *J Bone Joint Surg* 89-B:672–685.
36. Kim Y-J, Sah RLY, Doong J-YH, et al. 1988. Fluorometric assay of DNA in cartilage explants using Hoechst 33258. *Anal Biochem* 174:168–176.
37. Farndale RW, Buttle DJ, Barrett AJ. 1986. Improved quantitation and discrimination of sulphated glycosaminoglycans by use of dimethylmethylene blue. *Biochem Biophys Acta* 883:173–177.
38. Maffulli N, Longo UG, Franceschi F, et al. 2008. Movin and Bonar scores assess the same characteristics of tendon histology. *Clin Orthop Relat Res* 466:1605–1611.
39. Cook JL, Feller JA, Bonar SF, Khan KM. 2004. Abnormal tenocyte morphology is more prevalent than collagen disruption in asymptomatic athletes' patellar tendons. *J Orthop Res* 22:334–338.
40. Taylor SE, Smith RK, Clegg PD. 2007. Mesenchymal stem cell therapy in equine musculoskeletal disease: scientific fact or clinical fiction? *Equine Vet J* 39:172–180.
41. Foland J, Trotter GW, Powers BE, et al. 1992. Effect of sodium hyaluronate in collagenase-induced superficial digital flexor tendinitis in horses. *Am J Vet Res* 54:2371–2376.
42. Gaughan EM, Gift LJ, De Bowes RM, et al. 1995. The influence of sequential intratendinous sodium hyaluronate on tendon healing in horses. *Vet Comp Orthop Traum* 8:40–45.
43. Gift LJ, Gaughan EM, DeBowes RM, et al. 1992. The influence of intratendinous sodium hyaluronate on tendon healing in horses. *Vet Comp Orthop Traumatol* 4:151–157.
44. Tkach LV, Nguyen V-DN, Coutts RD, McFadden PR. 1993. A model of experimentally induced tendinosis. *Trans 39th Ann Mtg ORS* 18:366–366.
45. Soslowky LJ, Carpenter JE, DeBano CM, Banerji I, et al. 1996. Development and use of an animal model for investigations on rotator cuff disease. *J Shoulder Elbow Surg* 5:383–392.
46. Spurlock GH, Spurlock SL, Parker GA. 1988. Ultrasonographic, gross, and histologic evaluation of a tendinitis disease model in the horse. *Vet Rad* 30:184–188.
47. Williams IF, McCullagh GD, Goodship AE, et al. 1984. Studies on the pathogenesis of equine tendinitis following collagenase injury. *Res Vet Sci* 36:326–338.
48. Williams IF, McCullagh KG, Silver IA. 1984. Distribution of types I and III collagen and fibronectin in the healing equine tendon. *Connect Tissue Res* 12:211–227.
49. Silver IA, Brown PN, Goodship AE, et al. 1983. A clinical and experimental study of tendon injury and treatment in the horse. *Equine Vet J Suppl* 1–35.
50. Guest DJ, Smith MR, Allen WR. 2008. Monitoring the fate of autologous and allogeneic mesenchymal progenitor cells injected into the superficial digital flexor tendon of horses: preliminary study. *Equine vet J* 40:178–181.
51. Abrahamsson S-O. 1997. Similar effects of recombinant human insulin-like growth factor-I and II on cellular activities in flexor tendons of young rabbits: experimental studies in vitro. *J Orthop Res* 15:256–262.
52. Murphy DJ, Nixon AJ. 1997. Biochemical and site-specific effects of insulin-like growth factor I on intrinsic tenocyte activity in equine flexor tendons. *Am J Vet Res* 58:103–109.
53. Goodrich LR, Hidaka C, Strassheim ML, et al. 2004. Enhanced survival of chondrocytes over-expressing insulin-like growth factor I in equine cartilage repair. *Mol Ther* 9:S340.
54. Fortier LA, Smith RK. 2007. Evidence for stem cells in cartilage regeneration. *AAEP* 53:329–334.