Optimized Repopulation of Tendon Hydrogel: Synergistic Effects of Growth Factor Combinations and Adipose-Derived Stem Cells

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Abstract

Background: Tendon-derived extracellular matrix (ECM) hydrogel has been shown to augment tendon healing in vivo. We hypothesized that reseeding of the gel with adipose-derived stem cells (ASCs) could further assist repopulation of the gel and that combinations of growth factors (GFs) would improve the survival of these cells after reseeding. **Methods:** A tendon-specific ECM solution was supplemented with varying concentrations of basic fibroblast growth factor (bFGF), insulin-like growth factor-I (IGF-I), and platelet-derived growth factor-BB (PDGF-BB). Gels were then seeded with ASCs transfected with a green fluorescent protein/luciferin construct. Cell proliferation was determined using the MTT assay and histology, and GF and ASC augmented gels were injected into the back of Sprague Dawley rats. Bioluminescence of seeded gels was continuously followed after reseeding, and cell counts were performed after the gels were explanted at 14 days. **Results:** Synergistic effects of the GFs were seen, and an optimal combination was determined to be 10 ng/mL bFGF, 100 ng/mL IGF-I, and 100 ng/mL PDGF-BB (2.8-fold increase; $P < .05$). In vivo bioluminescence showed an improved initial survival of cells in gels supplemented with the optimal concentration of GF compared with the control group (10.6-fold increase at 8 days; $P < .05$). Cell counts of explants showed a dramatic endogenous repopulation of gels supplemented by GF + ASCs compared with both gels with GF but no ASCs (7.6-fold increase) and gels with ASCs but no GF (1.6-fold increase). **Conclusion:** Synergistic effects of GFs can be used to improve cellular proliferation of ASCs seeded to a tendon ECM gel. Reseeding with ASCs stimulates endogenous repopulation of the gel in vivo and may be used to further augment tendon healing.

Keywords: hydrogel, ECM, flexor tendon, reconstructive hand surgery, tissue engineering

Introduction

Rapid and strong tendon healing is important in reconstructive hand surgery, orthopedic surgery, and sports medicine. Enthesopathies are commonly diagnosed after overuse in the rotator cuff, Achilles tendon, medial and lateral epicondyle of the elbow, as well as other chronic disorders of the tendon-bone attachment. Biochemical changes in the tendon tissue and subsequent decreased biomechanical material properties lead to accumulative microscopic tears that may ultimately result in full-thickness tears.³⁰ The microstructural changes seen include micro-tears, thinning and disorganization of collagen fibers,^{15,24} neovascularization of the tendon matrix,²⁰ and morphological changes to tenocytes.³ Due to the high incidence of these conditions and their relatively poor clinical outcomes, various methods that have been attempted to augment and stimulate

healing²⁵ include injections of platelet-rich plasma (PRP),^{5,47,39} whole blood,¹⁹ or growth hormones,^{1,19,22} and the addition of multipotent stem cells.^{10,31,32}

We have previously characterized a hydrogel consisting of extracellular matrix (ECM) from human cadaver tendons¹² and have shown that healing of a partial thickness tendon wound can be accelerated by direct delivery to the zone of injury.²¹ We believe that this is accomplished through

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facilitated in situ regeneration by means of 3-dimensional guided tissue regeneration¹⁶ where migration of surrounding cells into the scaffold microenvironment is stimulated by chemical and biological cues, specific for tendon ECM, found in the gel. These cells would subsequently proliferate and replace the scaffold with regenerated tissue. The application of a biodegradable tendon-specific scaffold is attractive as it may act both as a structural matrix and as a 3-dimensional carrier of cells or growth hormones, which may further augment healing.^{14,28,40,50}

Several growth factors (GFs), including insulin-like growth factor-1 (IGF-1), platelet-derived growth factor-BB (PDGF-BB), and basic fibroblast growth factor (bFGF), have been shown to play important roles in normal tendon development and tendon wound healing. Key functions have been to promote tenocyte proliferation and ECM formation.^{6,7,11,45} Supplementation with various soluble GFs has therefore been used as a widely applied strategy to enhance scaffold properties and has shown considerable functional value for tissue engineering of tendon tissue.³⁷ Optimal supplementation strategies may thus be a valuable tool in tendon tissue engineering, both to increase cell survival and revitalization of the grafts prior to reconstructive use, but also to increase the speed of host cell recruitment into the scaffold in vivo.³⁸

The purpose of these experiments was thus to optimize proliferation of adipose-derived stem cells (ASCs) in a tendon-specific ECM hydrogel, using GF supplementation. The effects of individual GFs on their proliferation in vitro were compared. Also, the synergistic effect of IGF-1, PDGF-BB, and bFGF in combination was investigated. We hypothesized that, first, individual supplementation with IGF-1, PDGF-BB, and bFGF would increase proliferation of an ASC cell population. Second, we hypothesized that supplementation with combinations of IGF-1, PDGF-BB, and bFGF would produce a synergistic effect on cell proliferation and, third, that the optimal combination of factors would augment the repopulation of the tendon hydrogel in vivo and speed the process of tissue regeneration through ingrowth of host cells.

Materials and Methods

Chemical Decellularization

Human flexor digitorum profundus (FDP), flexor digitorum superficialis (FDS), and flexor pollicis longus (FPL) tendons were harvested from fresh-frozen cadaveric forearms (Science Care, Phoenix, Arizona). Epitenon, synovial sheath, and muscle tissue were meticulously debrided. Distally, FDS tendons were transected 2 cm proximal to the chiasma, and FDP and FPL tendons were transected 1 cm proximal to the osteotendinous junction. The tendons were then decellularized following a previously reported protocol.³⁶ The frozen

decellularized material was lyophilized (FreeZone Freeze Dryer; Labconco, Kansas City, Missouri) and milled into a fine powder using a Wiley Mini Mill (Thomas Scientific, Sedesboro, New Jersey). The powder was stored at 4°C until needed for use.

A DNA assay along with routine hematoxylin-eosin (H&E) histology and SYTO green fluorescent nucleic acid staining was used to quantify the effectiveness of decellularization of tendons. This is described in detail in a previous publication.³⁶ In short, DNA was extracted from lyophilized tendons using a DNeasy kit (QIAGEN, Valencia, California). The concentration of the extract was determined using an ultraviolet spectrophotometer (Biophotometer 22331; Eppendorf, Valencia, California) at a wavelength of 260 nm. The concentration of the samples was calculated by software on the spectrophotometer using a known extinction coefficient for dsDNA.

The ECM material was enzymatically digested by adding a 1 mg/mL solution of pepsin (Sigma, St Louis, Missouri) in 0.02 M HCl such that the final concentration of material was 20 mg/mL (2%, dry weight). The material was digested for up to 24 hours at room temperature with constant stirring. The liquid was checked for pH and homogeneity under a microscope after 24 hours to ensure complete digestion. While cooled on ice, the pepsin activity was completely inactivated by bringing the pH to greater than 8 before the final solution pH was neutralized to a pH of 7.4, and salt concentration was adjusted with the addition of 0.2M NaOH (1/10 of original digest volume) and 10× phosphate-buffered saline (1/10 of the final neutralized volume).

Lentivirus Production

To positively identify in vitro reseeded cells, candidate seeded cells were labeled with green fluorescent protein (GFP) using a lentiviral vector. pUbi-luc2-eGFP plasmids were constructed in a manner previously described.³⁴ High-titer lentiviral vectors were produced using a modified version of the protocol described by Zhang et al.⁵¹

Creation of GFP-Positive ASC Line

Fat pads from the groins of Sprague Dawley rats were harvested and dissected free of connective tissue. The fat pads were then immediately minced and stem cells (ASCs) were expanded in ASC basal medium (ASC-BM; Lonza, Basel, Switzerland) augmented with 10% fetal calf serum (FCS; Gibco, Grand Island, New York) using a technique published by Zuk et al.⁵² The cells were transduced with lentivirus carrying an ubiquitin promoter driving a bifusion reporter of firefly luciferase reporter gene (luc2) and an enhanced GFP (eGFP) gene at a multiplicity of infection of 50. The genetically modified ASC (ASC-luc2-eGFP) underwent 2 rounds of fluorescence-activated cell sorting

(FACSAria III; Becton Dickinson, San Jose, California) and were used for both cell culture and graft reseeding experiments.

GF Supplementation of Gels and Reseeding

Each condition was studied in triplicate, and experiments were repeated a minimum of 3 times.

Experiment 1: Evaluation of individual GFs in enhancing cell proliferation. ECM solution, with or without human bFGF, IGF-1, and PDGF-BB (PeproTech, Rocky Hill, New Jersey), was prepared. The concentrations studied were as follows: bFGF at 1, 5, and 10 ng/mL; IGF-1 at 10, 50, and 100 ng/mL; and PDGF-BB at 10, 50, and 100 ng/mL. Reseeding of the each solution preparation was performed by mixing 3×10^4 (ASC-luc2-eGFP) cells/0.2 mL ECM solution and then placing it in 48-well plates. Penicillin-amphotericin B (Gibco, Grand Island, New York) and 10% FCS were added to all solutions. The final mixture was then allowed to gel for 20 to 60 minutes at 37°C. Cell culture medium was gently added after gelation and changed every other day. Cells were stained using a live/dead assay using the manufacturer's protocol (Invitrogen, Valencia, California) on day 5 to check for viability on the gel surface and inside the gel using Nikon TS100 (Nikon, Melville, New York) at 40 $\times$. The position of the grid was not moved between photographs of live and dead cells to ensure that the 2 frames were from the same area of the specimen.

After 72 hours, the CellTiter assay (Promega, Fitchburg, Wisconsin) was used to determine cellular proliferation. All medium but 100 μ L was pipetted off each well, and MTS reagent was mixed with the gel, prior to standard incubation. Thereafter, the mixture was transferred to a microcentrifuge tube and spun down. Then 100 μ L supernatant was transferred to a 96-well plate for optical density reading at 490 nm.

Each experiment was performed in triplicates and 3 times; in total, 9 samples for each concentration were evaluated.

Experiment 2: Evaluation of synergy among bFGF, IGF-1, and PDGF-BB. Based on the results from experiment 1, the GFs were studied together to determine any synergistic effects. Each GF was studied at 3 different concentrations: bFGF at 0, 5, and 10 ng/mL; IGF-1 at 0, 50, and 100 ng/mL; and PDGF-BB at 10, 50, and 100 ng/mL. The number of different combinations of concentration was limited to 6 + 2 controls due to practical reasons. Groups not including FCS but with cells and gel as well as groups not including gel yielded low proliferation in experiment 1, and these groups were therefore omitted in experiments 2 and 3. Proliferation at 72 hours was determined using the same assay (MTT) as described above. To assess the impact of FCS on cell proliferation in the gel, all experiments were conducted with or without FCS.

Each experiment was performed in triplicates and 3 times; in total, 9 samples for each concentration were evaluated.

Experiment 3: In vivo. Eight Sprague Dawley rats (mean weight 280 g) were used as recipients for gel injections. The rats were kept anesthetized with isoflurane in a prone position. Dorsal hair was shaved and the animals were marked with a permanent marker to assign 6 injection sites. Under sterile conditions, 1 mL of a 2% gel solution supplemented with or without GF and ASCs was injected subcutaneously with a 25G needle and syringe. The corresponding groups are described in Figure 4. After the injections, all animals were housed at 21°C in a 12-hour light and dark cycle and were given food and water ad libitum.

Bioluminescence Imaging (BLI): Ex Vivo and In Vivo Cell Viability

Cell viability of ASC-luc2-eGFP seeded gels was monitored every other day, from day 2 to day 14 postoperatively, using BLI by the Xenogen Spectrum imaging system (Caliper Life Sciences, Mountain View, California). D-luciferin (Stanford Center for Innovation in In-Vivo Imaging, Stanford, CA, USA) was delivered via intraperitoneal injection (150 mg/kg) as the animals were temporarily anesthetized with isoflurane and positioned prone in the in vivo imaging system (IVIS) chamber. An imaging sequence was acquired in 7 segments with a 5-minute time delay (total 30 minutes per sequence) using Live Image Software for IVIS (Caliper Life Sciences) immediately after the rats were injected. Field of view was set to image the whole animal at once. Identical settings were used to acquire each image. To quantify measured light, regions of interest were drawn over the seeded scaffolds and the maximum radiance (photons/cm²/s/steradian) was obtained, as validated previously.³⁵ Steady-state peak radiance readings were compared.

Histology

Gel specimens were harvested at 14 days and were used for histology. Formalin-fixed, paraffin-embedded sections (5 μ m) from the mid-substance portion of each gel were stained. H&E (Sigma) was used for assessment scaffold morphology and Vectashield (Vector Labs, Valencia, California) mounting medium with propidium iodide (PI) for cell counts of viable cells within the gels. All histological images were taken with an inverted microscope (Nikon TS100; Nikon).

Cell Counts

Using PI and matched H&E sections for anatomy, cell counts were performed by 2 independent researchers. Eight random representative high-power field (hpf, 20 $\times$) images were centered over the central parts of the gels with a

Figure 1. (Left) MTT proliferation assays 5 days after reseeding: Impact of individual factors of bFGF, IGF-1, and PDGF-BB at 3 different concentrations. Three different control groups were used including (1) without (no) GF without (no) FCS, (2) a combination of growth factors 10 ng/mL bFGF, 100 ng/mL IGF-1, and 100 ng/mL PDGF-BB, without (no) FCS, and (3) without (no) GF with (+) FCS. (Right) MTT proliferation assays 5 days after reseeding with different combinations of factors (doses are in ng/mL in the order bFGF/IGF-1/PDGF-BB). Two different control groups were used including (1) without (no) GF without (no) FCS and (2) without (no) GF with FCS. Statistical significant changes between groups are presented for comparisons that were found relevant. Note. ns, nonsignificant difference; bFGF, basic fibroblast growth factor; IGF, insulin-like growth factor; PDGF-BB, platelet-derived growth factor–BB; FCS, fetal calf serum.

*Significant difference at $P < .05$.

**Significant difference at $P < .005$.

superscript grid, to ensure that the evaluated area examined remained the same in all samples.

Statistical Analysis

Data were reported as mean $\pm$ standard deviation (SD). Comparisons across groups were performed using the paired Student t test. One-way analysis of variance for repeated measures with Sidak's multiple comparisons test was performed to test whether changes attributed to different GF concentrations were significant. Statistical calculations were done using GraphPad Prism version 6.0 for Mac OS X (GraphPad Software, San Diego, California; www.graphpad.com). For all analyses, probabilities of less than .05 were accepted as significant.

Each in vitro experiment was performed a minimum of 3 times for each cell type and condition. Each data point was measured in triplicate. In vivo experiments were performed on 8 rats.

Results

Assessment of Cellular Proliferation

A linear standard curve was created for the ASCs used (ASC-luc2-eGFP), validating the use of the CellTiter assay for our cell line (data not shown). In addition, the results of

the CellTiter assay proved to be independent of the presence of bFGF, IGF-1, and PDGF-BB, showing that the GFs did not alter the metabolism of the cells in a way that altered the assay.

Experiment 1: Evaluation of individual GFs in enhancing cell proliferation. Within each GF, the concentration that produced the highest proliferation was 10 ng/mL bFGF, 100 ng/mL IGF-1, and 100 ng/mL PDGF-BB (Figure 1). PDGF-BB exhibited a dose-dependent significant effect of proliferation that was not seen with bFGF or IGF-1, with significantly higher proliferation with 100 ng compared with both other doses. With bFGF, there was no significant difference between the different doses ($P < .05$). With IGF-1, 100 ng/mL produced significantly larger proliferation than 50 ng/mL ($P = .04$), whereas it was not significantly higher than 10 ng/mL; 100 ng/mL of PDGF-BB resulted in the most evident increase in proliferation over the control (no FCS and no GF; 3.4-fold increase; $P < .01$). A higher dose of PDGF-BB (200 ng/mL) was also tested to make sure the proliferation rate plateaued after 100 ng/mL. No significant increase in proliferation was seen with the higher dose (3.5-fold increase; $P = .8$); therefore, 100 ng/mL was chosen for all further experiments.

The addition of GFs (at a concentration of 10 ng/mL bFGF, 100 ng/mL IGF-1, and 100 ng/mL PDGF-BB) but no FCS produced a significantly higher proliferation (1.13-fold

Figure 2. Live/dead stain of ASCs reseeded in hydrogel after 5 days in vitro, without serum (left 2 columns), and with serum (right 2 columns).

Note. Three different concentrations of GFs: (1) no GF, (2) 5/50/100 (bFGF/IGF-1/PDGF-BB ng/mL), and (3) 10/100/100 (bFGF/IGF-1/PDGF-BB ng/mL; 40×). ASC, adipose-derived stem cell; GF, growth factor; bFGF, basic fibroblast growth factor; IGF, insulin-like growth factor; PDGF-BB, platelet-derived growth factor–BB.

increase; $P = .04$) than gels with no GF and no FCS. When FCS was added alone (without GF), the increase was 1.75-fold ($P = .001$) compared with control gels (no GF and no FCS) and 1.55-fold compared with gels with an optimal concentration of GF (10 ng/mL bFGF, 100 ng/mL IGF-1, and 100 ng/mL PDGF-BB) but no FCS. This is especially interesting as it highlights the effects of addition of FCS in relation to the addition of GFs.

Experiment 2: Evaluation of synergy among bFGF, IGF-1, and PDGF-BB. Synergistic effects were determined by studying a combination of each GF at 3 different concentrations: bFGF at 0, 5, and 10 ng/mL; IGF-1 at 0, 50, and 100 ng/mL; and PDGF-BB at 10, 50, and 100 ng/mL (Figure 2). The most effective and efficient combination proved to be 10 ng/mL bFGF, 100 ng/mL IGF-1, and 100 ng/mL PDGF-BB (2.95-fold increase; $P < .001$), compared with 0 ng/mL bFGF, 100 ng/mL IGF-1, and 100 ng/mL PDGF-BB that increased 2.7-fold ($P < .005$) and 5 ng/mL bFGF, 50 ng/mL IGF-1, and 50 ng/mL PDGF-BB that increased 2.5-fold ($P < .005$). Statistical analysis was performed to evaluate

significant changes between the respective groups and the concentration 10 ng/mL bFGF, 100 ng/mL IGF-1, and 100 ng/mL PDGF-BB was found to produce the strongest proliferative response. This optimal concentration was used for the remainder of the study.

Experiment 3 In vivo. Continuous in vivo BLI (Figure 3) was used to quantify total flux corresponding to viable luciferase + cells over 14 days. Gels 1, 3, and 5 correspond to the gels that included luciferase + cells. All gels that included luciferase + cells (gels 1, 3, and 5) showed an increase in flux from days 4 to 8 indicating the proliferation of seeded cells in the gels. The highest increase was seen in gel 1 (gel + GF + FCS + ASC), followed by gel 3 (gel + FCS + ASC), and gel 5 (gel + ASC). Only gels 1 and 3, however, showed a statistically significant increase in flux from days 4 to 8 ($P < .005$, for both).

From days 8 to 14, all gels containing luciferase + cells displayed a decrease in flux, indicating a decline in the number of viable cells. No viable, seeded luciferase + ASCs were detected in any of the gels by day 14. Gels 2, 4, and 6 showed no signal at any time point.

Figure 3. In vivo ASC viability and reseeded in vivo.

Note. (a) Bioluminescence imaging was used to follow luciferase + cells delivered in hydrogels in the back of rats. This is an example of a representative rat at 8 days. (b) Numbered injections: 1, gel + GF + FCS + ASC; 2, gel + GF + FCS; 3, gel + FCS + ASC; 4, gel + FCS; 5, gel + ASC; 6, gel. Only gels 1, 3, and 5 included cells that were luciferase +. (c) Quantification of total flux over 14 days. 1, 3, and 5 correspond to the gels that included luciferase + cells. Gels 2, 4, and 6 showed no signal at any time point. ASC, adipose-derived stem cell; GF, growth factor; FCS, fetal calf serum; IGF, insulin-like growth factor; PDGF-BB, platelet-derived growth factor-BB.

*Significant difference in flux ($P < .05$) compared with control (control = gel 5 that included cells but no GF or FCS).

Results seen in H&E-stained and corresponding PI-stained sections of explanted gels are displayed in Figure 4. Based on bioluminescence data from day 14, we concluded that all cells found in the explant were cells that had migrated into the gels. Cell counts from consecutive sections (Figure 5) were therefore used to evaluate ingrowth of host cells into the gel at 14 days. The highest average numbers of cells were found in gels that included both FCS and cells (gel 1 [gel + GF + FCS + ASC]: 1195 ± 102 and gel 3 [gel + FCS + ASC]: 738 ± 26), whereas gels that included FCS but no cells showed significantly lower cell counts (gel 2 [gel + GF + FCS]: 158 ± 44 and gel 4 [gel + FCS]: 167 ± 43), regardless of if they were supplemented with GF or not. Gels that were supplemented with cells but no FCS (gel 5 [gel + ASC]: 84 ± 42) as well as gels without any supplementation (gel 6 [gel]: 69 ± 28) showed even lower counts.

Discussion

The concept of revitalization of biostatic allografts has proven promising when novel treatments for functional regeneration of tendon tissue have been explored.^{6,7,33,49} In this study, we show how cell proliferation and cell adhesion can be improved in a tendon-specific ECM hydrogel by addition of GFs, FCS, and ASCs. Also, we demonstrate how ASCs can be used to stimulate better biointegration

through recruitment of host cells in vivo. These results suggest that the addition of GFs and ASCs may be used to increase the therapeutic effect of this hydrogel at a tendon injury site by faster repopulation and remodeling. Furthermore, the increased cellularity may increase synthetic and contractile ability, thereby increasing construct strength and stiffness.² This property however remains to be proven in a tendon injury model in vivo.

The tendon-specific ECM hydrogel has been thoroughly described previously in terms of proteomics, rheology, ultrastructure, and biocompatibility,¹² and has also been shown to augment healing of tendon tissue in a novel and reproducible Achilles tendon injury model, producing stronger tissue with a more mature collagen structure.²¹ The benefits observed are likely because it contains not only tendon matrix but also its associated proteins and biological cues, providing a preferred environment or blueprint for cellular migration and proliferation. As an injectable scaffold material, it therefore holds potential to be a useful method of augmenting tendon healing in cases of traumatic and attritional tendon injury. Thus, in a clinical setting, injection of this gel, augmented by GFs or the patients' own ASCs, could hypothetically improve patient outcomes by accelerating the early healing processes and allowing for earlier mobilization of the injured tendon. It may further be hypothesized that this process could be mediated by earlier

Figure 4. The 14-day explants (1, gel + GF + FCS + ASC; 2, gel + GF + FCS; 3, gel + FCS + ASC; 4, gel + FCS; 5, gel + ASC; 6, gel). Note. The left column (1a, 3a, 5a, 2a, 4a, 6a) is H&E stained and the right (1b, 3b, 5b, 2b, 4b, 6b) is corresponding PI stained. All is 20 $\times$. GF, growth factor; FCS, fetal calf serum; ASC, adipose-derived stem cell; H&E, hematoxylin-eosin.

Figure 5. Cell counts (cells/high-power field) in gels after explant at 14 days in vivo (1, gel + GF + FCS + ASC; 2, gel + GF + FCS; 3, gel + FCS + ASC; 4, gel + FCS; 5, gel + ASC; 6, gel). Note. GF, growth factor; FCS, fetal calf serum; ASC, adipose-derived stem cell.

engraftment, which is initiated by enhanced intrinsic repair rather than extrinsic tendon healing.^{26,27,49} Intrinsic healing is driven by a GF-induced increased expression of tendon-associated structural protein genes such as collagen I, collagen III, decorin, scleraxis B, and tenascin C, which leads to an increased collagen and protein synthesis.⁶ Analysis of tendon-associated gene expression and in-depth analysis of collagen deposition and maturation of the gel matrix of the respective groups were not performed in the present study. This was because the main scope was to address optimization of gel seeding and proliferation in vitro and in vivo, and because the temporal resolution of the study design limited the possibility to assess such long-term effects of seeded gels in the tissue.

Although there are a variety of GFs involved in the tendon healing process, FGF, IGF, and PDGF were chosen specifically. First, FGF is upregulated after tendon injuries,⁹ which leads to tendon fibroblast proliferation and increased collagen production.^{8,45} In vivo delivery of FGF by viral and nonviral vectors has also shown increased collagen production and overall increased tendon strength.^{17,18,42,43,48} IGF is also expressed after flexor tendon injuries^{4,29} and is expressed by nearly all tendon fibroblasts.⁴⁶ Last, PDGF is known to increase DNA synthesis⁴ and stimulate proliferation and migration of tendon fibroblasts.⁴³ In vivo studies

have also shown that long-term delivery of PDGF improves Achilles tendon healing in a rat model⁴¹ and flexor tendon healing in a dog model.⁴⁴

The enhanced proliferation of ASCs that was observed with all individual GFs showed an improved effect when used in combinations. This synergistic effect of the factors can be found in other studies when combinations of these GFs were used in decellularized flexor tendon reseeding,^{6,7,11,37,45} indicating that GF supplementation may be a potential strategy to increase the regenerative effect in vivo. The use of bFGF and IGF-1 showed a moderate improvement when compared with the control where no clear dose-dependent increase was seen with higher concentrations. PDGF-BB however displayed a significant increase in proliferation in a dose-dependent manner and was the most potent independent stimulator for increased proliferation with a plateau at 100 ng. This was consistent with results found in both rabbit and human tenocytes.^{11,37} Based on these findings, we found no reason for testing a wider range of concentrations and decided to concentrate test on synergistic effects on the concentrations that showed the highest proliferative effect. When we combined 2 or more GFs, we observed a synergistic effect on proliferation, and this was significantly higher when all 3 GFs were added at a higher concentration.

In vivo BLI showed that ASCs delivered in hydrogels that included GF remained viable longer and demonstrated enhanced reseeding efficiency than those delivered without GF (30% increased). BLI also demonstrated how this function could be greatly improved when FCS was included in the gel (10-fold increase). After 8 days, a decline in cell number was seen, and most replanted cells were not detectable by day 14, indicating that most of the engrafted cells were lost. This is a pattern that has been shown previously in various stem cell lineages in other healing models involving hydrogels.^{13,23,38} In a similar study on mesenchymal stem cell (MSC) reseeded ECM hydrogel in skin wound healing, MSC viability gradually diminished over 14 days in all conditions that were tested. It was however concluded that MSCs delivered in a hydrogel significantly increased the number of host cells present throughout wound healing.³⁸ Also, and analogous with our study, it was suggested that enhanced stem cell survival in the first 2 weeks postimplantation is sufficient to promote accelerated wound healing and that long-term engraftment of cells may not be necessary. The limited cell survival after 14 days can be explained by hypoxia and nutrient deprivation within the hydrogel, as cells proliferate in isolated pockets throughout the gel. Alternatively, it is possible that the tendon hydrogel lacks native cellular cues found in adipose tissue, required for ASC proliferation. Also, the release profile of the GFs from the gel is unknown. As the GFs are simply mixed into the gel, it is possible that they undergo burst release and are therefore not present in the gel for the duration of the

experiment. In-depth analysis of the release profile of GFs and other factors that enhance cell survival is therefore warranted in future studies. Also, further studies may be directed to further address the specific benefits of the ECM hydrogel in relation to other gel substitutes such as matrigel or fibrin glue.

Cell counts of explants showed in our study signs of dramatic endogenous repopulation of gels supplemented by GF + ASCs compared with both gels with GF but no ASCs (7.6-fold increase) and gels with ASCs but no GF (1.6-fold increase) (Figure 5). Also important is the finding that FCS is crucial to cell survival in this hydrogel-cell system, both in vitro (Figure 2) as well as in vivo. Although FCS is a common reagent used in in vitro studies, it may be less acceptable in a clinical setting. A more acceptable surrogate, concentrated patient serum/plasma, may be used instead.

A shortcoming in this study was however that only day 14 was used as a time point to histologically assess ingrowth of cells in relation to seeded cells. Additional experiments using immunohistochemistry on GFP positive seeded cells at various time points would potentially have added additional temporal information on cell ingrowth and death of seeded cells.

This animal model has potential in forthcoming studies to elucidate various key questions in guided tissue regeneration stimulated by tissue-engineered tendon-specific ECM hydrogels. Upcoming studies will be focused on regenerative potential in various cells harvested by the recipient, such as autografted ASCs and PRP that could work as in situ providers of the above-described GFs. The influence of ASC reseeded gels in tendon healing will also be addressed in an in vivo tendon defect model, where the degradation kinetics of the scaffold can be addressed as well as evaluation of the matrix that has been formed within the tendon tissue.

Conclusion

Tendon healing through guided tissue regeneration is mediated by migration of host cells to the injured area and cellular proliferation at that site. In this article, we have shown how a tendon-specific ECM hydrogel supplemented with GFs stimulates cellular proliferation within the gel and how host cell infiltration into the gel is increased with an optimal concentration of GFs in vivo. Future studies will evaluate how these findings can be used to stimulate healing in an in vivo tendon defect model. In the future, this tendon-specific ECM hydrogel may be used as a therapeutic agent in chronic and acute tendon injuries, with or without augmentation with GFs and stem cells.

Authors' Note

This article was presented at the American Association for Hand Surgery (AAHS) Annual Meeting, January 21-24, 2015, Bahamas. All authors have been personally and actively involved in

substantive work leading to the report and will hold themselves jointly and individually responsible for its content.

Ethical Approval

All experiments were approved by the local Committee of Laboratory Animal Ethics (Institutional Animal Care and Use Committee).

Statement of Human and Animal Rights

Animals were handled in accordance with the Principles of Laboratory Animal Care.

Statement of Informed Consent

Informed consent was obtained when necessary.

Declaration of Conflicting Interests

The authors declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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