Published in final edited form as:
J Biomed Mater Res A. 2014 May ; 102(5): 1275–1285. doi:10.1002/jbm.a.34789.

Sustained delivery of bioactive TGF-β1 from self-assembling peptide hydrogels induces chondrogenesis of encapsulated bone marrow stromal cells

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Abstract

Tissue engineering strategies for cartilage defect repair require technology for local targeted delivery of chondrogenic and anti-inflammatory factors. The objective of this study was to determine the release kinetics of transforming growth factor β1 (TGF-β1) from self-assembling peptide hydrogels, a candidate scaffold for cell transplant therapies, and stimulate chondrogenesis of encapsulated young equine bone marrow stromal cells (BMSCs). Although both peptide and agarose hydrogels retained TGF-β1, 5-fold higher retention was found in peptide. Excess unlabeled TGF-β1 minimally displaced retained radiolabeled TGF-β1, demonstrating biologically relevant loading capacity for peptide hydrogels. The initial release from acellular peptide hydrogels was nearly 3-fold lower than agarose hydrogels, at 18% of loaded TGF-β1 through 3 days as compared to 48% for agarose. At day 21, cumulative release of TGF-β1 was 32–44% from acellular peptide hydrogels, but was 62% from peptide hydrogels with encapsulated BMSCs, likely due to cell-mediated TGF-β1 degradation and release of small labeled species. TGF-β1 loaded peptide hydrogels stimulated chondrogenesis of young equine BMSCs, a relevant preclinical model for treating injuries in young human cohorts. Self-assembling peptide hydrogels can be used to deliver chondrogenic factors to encapsulated cells making them a promising technology for in vivo, cell-based regenerative medicine.

Keywords

Tissue Engineering; Sustained Delivery; Bone Marrow Stromal Cell; Regenerative Medicine; Cartilage Repair

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Introduction

Insufficient endogenous repair and regeneration of articular cartilage defects results in a compromised tissue incapable of performing its physiologic load bearing function and ultimately leads to the painful pathology, osteoarthritis. The limited healing capacity of articular cartilage has motivated the development of numerous tissue-engineering approaches which combine a chondrogenic cell source with a biocompatible scaffold and differentiation and tissue production factors. Despite recent promising results utilizing bone marrow derived stromal cells (BMSCs)[1], no treatment has succeeded in producing hyaline differentiated tissue that fully integrates with the surrounding native cartilage and does not produce inflammation, senescence, apoptosis, or necrosis[2]. To develop a therapy that overcomes these challenges, local delivery of chondrogenic factors will likely be of key importance[2].

Transforming growth factor β1 (TGF-β1) has been extensively used to promote chondrogenesis in cartilage tissue engineering applications[3–6]. However, these in vitro studies predominantly delivered TGF-β1 via supplemented medium. To translate these results for use in vivo, TGF-β1 has been formulated for sustained release by exploiting its affinity for a variety of materials including heparin[7,8], gelatin[9,10], fibrin[11,12] and modified dextran[13]. While these hydrogels were able to deliver TGF-β1 to encapsulated cells, a scaffold with nanoscale dimensions and simple gelation may produce an improved chondrogenic microenvironment and increase clinician ease of use.

Self-assembling peptide hydrogels[14–16] are a unique class of peptides that form three-dimensional scaffolds at physiologic pH and ionic strength[17]. The resulting tissue engineering matrix contains nanofibers with the same length scale as native extracellular matrix,[18] is biocompatible for in vivo use[19,20], and has low immunogenic and pathogenic risk[21]. In addition, these self-assembling peptides have the capacity to deliver small molecules[22], functional proteins[20,23,24], therapeutic macromolecules[15], and bioactive motifs[14].

The objective of this study was to determine the release kinetics of TGF-β1 from self-assembling peptide hydrogels and use them to stimulate chondrogenesis of encapsulated young equine BMSCs, a clinically relevant cell source for injury repair in young human cohorts. Release of TGF-β1 from agarose hydrogels was characterized as a benchmark comparison for release from self-assembling peptide hydrogels. Agarose was chosen because, as a commonly used hydrogel for electrophoresis and chromatography of large biomolecules, it is uncharged and induces minimal protein adsorption and precipitation[25]. In addition, it has been used extensively to study the synthesis and accumulation of extracellular matrix synthesized by encapsulated BMSCs.

Materials and Methods

Materials

Self-assembling peptide with the sequence AcN-$(KLDL)_3$-CNH_2, subsequently $(KLDL)_3$ or simply peptide, was synthesized by the MIT Biopolymers Laboratory (Cambridge, MA) using an ABI Model 433A peptide synthesizer with FMOC protection. All other materials were purchased from the suppliers noted below.

TGF-β1 Uptake with Acellular Hydrogels

Acellular 0.35% (w/v) peptide solutions were cast using acellular agarose molds to initiate self-assembly, generating 50 μL initial volume, 6.35 mm diameter by 1.6 mm thick disks (one disk per well in 24-well plates) as described previously[26]. An identical geometric

configuration was used to cast disks of 2% low melting point agarose (Invitrogen, Carlsbad, CA). ^{125}I-TGF-β1 (55pM, 1.4 ng/mL, 3500 Ci/mmol, PerkinElmer, Waltham, MA) was either added to hydrogel solutions prior to gelation (encapsulated within hydrogels), or added to the equilibration bath. Where indicated, unlabeled TGF-β1 (10–100 ng/mL, R&D Systems, Minneapolis, MN) was mixed simultaneously with ^{125}I-TGF-β1. Hydrogels were incubated at 37°C with agitation in a bath consisting of high glucose DMEM (Invitrogen) with 1% ITS+1 (Sigma-Aldrich, St. Louis, MO), PSA (100 U/mL penicillin, 100 μg/mL streptomycin, and 250 ng/mL amphotericin), 10mM HEPES, L-proline, sodium pyruvate, and non-essential amino acids (equilibration bath). The equilibration bath was not changed for 5 days, after which it was collected and stored at −20°C. Hydrogel samples were rinsed 3x in PBS (30 sec/rinse) to remove surface-bound ^{125}I-TGF-β1 and then mechanically disrupted (acellular peptide) or melted (acellular agarose) to measure retained ^{125}I-TGF-β1. The ^{125}I-radioactivity of all equilibration bath and hydrogel samples were quantified individually using a gamma counter (model B5002, Packard Instrument Company, Meriden, CT). The uptake ratio was calculated as the concentration of the ^{125}I-TGF-β1 in the hydrogel samples (per intra-gel wet weight) normalized to the concentration of ^{125}I-TGF-β1 in the equilibration bath. Radio-labeled and unlabeled TGF-β1 were assumed to partition into the hydrogels in an identical manner[27]. To account for the presence of small labeled species accumulated during the time course of experimentation, Sephadex G25 chromatography of the equilibration bath (see below) was performed at day 5 and the fraction of small molecule ^{125}I species was determined[28]. The uptake ratio of free ^{125}I was also measured separately to correct for the presence of such free label.

Tissue Harvest

Equine bone marrow was harvested from the sternum and iliac crest of immature mixed-breed horses (2–4-month-old foals) as described previously[29]. Horses were euthanized at Colorado State for reasons unrelated to conditions that would affect marrow. Bovine bone marrow was harvested from newborn bovine calves (Research 87, Marlborough, MA) as described previously[26].

Cell Isolation

BMSCs were isolated from equine[29] and bovine[26] marrow via differential adhesion to separate BMSCs from the total nucleated cell population[5]. After reaching local confluence, BMSCs were cryopreserved and stored for future use. Prior to peptide hydrogel encapsulation, BMSCs were expanded by plating at 6×10^3 cells/cm^2 and culturing for three days in low glucose DMEM (Invitrogen), 10% ES-FBS (Invitrogen), 10mM HEPES and PSA plus 5 ng/mL bFGF (R&D Systems, Minneapolis, MN). After 3 days, cells were detached with 0.05% trypsin/1mM EDTA (Invitrogen) at ~3×10^4 cells/cm^2 (passage 1) and replated at 6×10^3 cells/cm^2. Passage 2 cells were used for 3D peptide hydrogel culture.

TGF-β1 Release from Acellular and BMSC-seeded Hydrogels

^{125}I-TGF-β1 was mixed with all hydrogel solutions prior to gelation. Acellular hydrogels were incubated in equilibration bath with the same composition as was used for uptake ratio measurements. Where indicated, 100 ng/mL unlabeled TGF-β1 was also mixed with hydrogels prior to gelation. BMSC-seeded hydrogels were cultured in chondrogenic medium which consisted of the equilibration bath formulation plus the following supplements: 0.1 μM dexamethasone (Sigma-Aldrich) and 37.5 μg/mL ascorbate-2-phosphate (Wako Chemicals, Richmond, VA). Chondrogenic medium or equilibration bath was changed every 2–3 days and conditioned samples were frozen at −20C. At days 7, 14, or 21, acellular hydrogels samples were collected as described above. For BMSC-seeded peptide hydrogels,

samples were digested with proteinase-K (Roche) to remove secreted ECM proteins and release ^{125}I-label.

^{125}I-TGF-β1 Chromatography

Immediately before use for all experiments, ^{125}I-TGF-β1 was purified by Sephadex G25 chromatography to remove small ^{125}I species that may result from time-dependent degradation of the label or incomplete purification as received from the supplier[28]. Sephadex G25 chromatography was performed with a 0.7 × 50 cm gravity fed column equilibrated in 1 M acetic acid supplemented with 0.1% BSA and 0.1% Triton X-100. Purified ^{125}I-TGF-β1 was collected in the void volume. This ^{125}I-TGF-β1 stock was added to equilibration bath (final concentration 1.4 ng/mL) and as a control an aliquot was incubated at 37°C for 7, 14, or 21 days and characterized by Sephadex G25 chromatography. For release experiments from either acellular or BMSC-seeded hydrogels, ^{125}I-containing species in the equilibration bath were characterized by Sephadex G25 chromatography at days 7, 14, and 21. ^{125}I-species retained within acellular hydrogels during release experiments were recovered by mechanical disruption of the gel and characterized by G25 chromatography. The void volume (K_{av}=0) and total volume (K_{av}=1) were calculated from the peaks for ^{125}I-TGF and free ^{125}I label released to the equilibration bath from acellular peptide at day 7 (Fig. 4B). Macromolecular species were defined as $-0.3<K_{av}<0.3$ and small molecule species were defined as $K_{av}>0.8$.

BMSC Chondrogenesis via Controlled TGF-β1 Delivery

BMSCs were encapsulated in $(KLDL)_3$ peptide hydrogels (0.35% w/v) at 10^7 cells/mL and cultured in chondrogenic medium with (Med-TGF) or without (TGF-free) 10 ng/mL unlabeled recombinant human TGF-β1 as positive and negative controls for chondrogenesis, respectively. In separate hydrogels, BMSCs were encapsulated in TGF-β1 adsorbed peptide hydrogels defined as 0.35% $(KLDL)_3$ solution mixed with either 10 or 100 ng/mL unlabeled TGF-β1 prior to gelation (Ads-TGF-10 or Ads-TGF-100, respectively). Ads-TGF-10 and Ads-TGF-100 hydrogels were cultured in TGF-β1-free medium. 750 μL of medium was added per hydrogel and medium was changed every 2–3 days for up to 21 days of culture. The total dose of TGF-β1 in the Med-TGF condition with 9 medium changes over 21 days was thus 67.5 ng as compared to 5 ng of TGF-β1 in the highest adsorbed TGF-β1 condition (Ads-TGF-100) i.e. 100 ng/mL in a 50 μL hydrogel.

DNA and ECM Biochemistry

During the last 24 hours of culture, medium was additionally supplemented with 5 μCi/mL of ^{35}S-sulfate and 10 μCi/mL of ^{3}H-proline to measure cellular biosynthesis of proteoglycans and proteins, respectively. Upon termination of culture, peptide hydrogels were rinsed 4×30 minutes in excess unlabeled sulfate and proline, weighed wet, lyophilized, weighed dry, and digested in 250 μg/mL proteinase-K (Roche Applied Science, Indianapolis, IN) overnight at 60°C. Digested samples were assayed for total DNA content by Hoechst dye binding[30], retained sulfated glycosaminoglycan (sGAG) content by DMMB dye binding assay[31], and radiolabel incorporation with a liquid scintillation counter. Conditioned culture medium collected throughout the study was also analyzed for sGAG content by DMMB dye binding. Percent sGAG retained is defined as: (hydrogel sGAG content)/(hydrogel sGAG content + cumulative sGAG in conditioned medium).

Statistical analysis

All data are presented as mean ± SEM. Data were analyzed with a mixed model of variance with animal donor as a random factor using SYSTAT version 12. Residual plots for dependent variable data were constructed to test for normal distribution. If this assumption

was not met, data were log transformed to ensure normality. Pairwise comparisons were made by *post hoc* Tukey tests with significance threshold set at $p<0.05$.

Results

TGF-β1 Uptake by Acellular Peptide and Agarose Hydrogels

To investigate delivery of TGF-β1 by peptide and agarose hydrogels, ^{125}I-TGF-β1 was added either to the equilibration bath (Fig. 1A) or to the hydrogel solution prior to gelation (Fig. 1B). Hydrogels were incubated with agitation at 37°C for 5 days without equilibration bath changes. The uptake ratio of ^{125}I-TGF-β1 (ratio of ^{125}I-TGF-β1 concentration in the gel to concentration in the equilibration bath) of equilibration bath loaded ^{125}I-TGF-β1 was 6-fold higher for peptide than for agarose hydrogels (18.5 ± 1.26 vs. 3.1 ± 0.17, respectively, Fig. 1A, $p<0.001$) and greater than the uptake of free ^{125}I label (1.3 ± 0.06 and 0.6 ± 0.03, in each hydrogel respectively, Fig. 1A, $p<0.001$). When ^{125}I-TGF-β1 was added to the hydrogel solution prior gelation, the uptake ratio was ~5-fold higher for both peptide and agarose hydrogels (85.8 ± 1.1 and 17.1 ± 0.4, respectively, Fig. 1B, $p<0.001$).

To investigate whether ^{125}I-TGF-β1 uptake by peptide and agarose hydrogels could be blocked by the addition of excess unlabeled TGF-β1, up to 100 ng/mL of unlabeled TGF-β1 was added simultaneously with 1.4 ng/mL of ^{125}I-TGF-β1 to peptide and agarose hydrogels prior to gelation. When 100 ng/mL of unlabeled TGF-β1 was added, the uptake ratio decreased by just 16% for peptide and 27% for agarose (Fig. 1B, $p<0.001$). For both peptide and agarose acellular hydrogels the wet weights ranged from 48.1±1.6 – 50.4±0.7 μg (mean ± sem) consistent with the nominal 50 μL gel volume and a density of 1 g/mL.

Release of TGF-β1 from Acellular Peptide and Agarose Hydrogels

^{125}I-labeled TGF-β1 was mixed with peptide or agarose prior to gelation without cells and the resulting hydrogels were maintained in TGF-β1-free equilibration bath. Independent experiments showed that the uptake ratio of ^{125}I-TGF-β1 did not change after 2 days of agitation at 37°C, indicating that 2 days was sufficient for the system to reach transport equilibrium (data not shown). Thus for release experiments, equilibration bath changes were conducted every 2–3 days for 21 days. Collected equilibration bath samples were analyzed for ^{125}I-TGF-β1 content (Fig. 2A). By day 3, 18% of the total ^{125}I-TGF-β1 loaded was released from peptide hydrogels, while 48% was released from agarose hydrogels. By the end of 21 days, TGF-β1 release had increased to 44% for peptide and 82% for agarose (Fig. 2B, $p<0.001$). At day 21, hydrogels were melted at 70°C and mechanically disrupted to measure retained ^{125}I-TGF-β1. Peptide hydrogels retained 56% of the total ^{125}I-TGF-β1 loaded versus 18% for agarose (Fig. 2B, $p<0.001$).

BMSC-Encapsulation within Peptide Hydrogels Increases TGF-β1 Release

Encapsulating BMSCs within peptide hydrogels altered the release profile of TGF-β1, consistently increasing the TGF-β1 release by approximately a factor of two throughout the 21-day timecourse. At day 3, 13%–16% ^{125}I-TGF-β1 release had occurred in acellular peptide hydrogels compared to 26%–28% in BMSC-seeded hydrogels (Fig. 3A). By days 7, 14, and 21, TGF-β1 release had increased to 25%, 27%, and 32% for acellular hydrogels compared to 48%, 59%, and 62% for BMSC-seeded hydrogels, respectively ($p<0.001$ for acellular vs. BMSC at each timepoint, Fig. 3C). Furthermore, replotting as TGF-β1 release per day (Fig. 3B) suggests BMSC-seeded hydrogels had an accentuated initial release compared to acellular hydrogels from days 0–8. However from days 11–21, TGF-β1 release per day from BMSC-seeded hydrogels was comparable to acellular hydrogels (Fig. 3B). The retained TGF-β1 content within peptide hydrogels was consistent with the release profiles

with 46% more TGF-β1 retained within acellular peptide than the BMSC-seeded peptide at day 7 and 76% more at days 14 and 21 (Fig. 3C).

While the acellular peptide hydrogel release experiments in Fig. 2 contained no unlabeled TGF-β1, the acellular peptide hydrogels for Fig. 3 were loaded with 100 ng/mL of unlabeled TGF-β1 in addition to 1.4 ng/mL of ^{125}I-TGF-β1. The addition of unlabeled TGF-β1 resulted in comparable retention and release of ^{125}I-TGF-β1 from acellular peptide with 68% retained and 32% released at day 21 (Fig. 3C) as compared to 56% retained and 44% released without unlabeled TGF-β1 (Fig. 2B). This is consistent with Fig. 1B where the addition of excess unlabeled TGF-β1 had a limited effect on the uptake of ^{125}I-TGF-β1.

Peptide Hydrogels Retained Macromolecular ^{125}I-Labeled Species

Prior to release and uptake experiments, ^{125}I-TGF-β1 was purified by Sephadex G25 size-exclusion chromatography to remove small labeled species. As a control, this purified macromolecular ^{125}I-TGF-β1 stock solution was incubated for 7, 14, or 21 days at 37°C in equilibration bath. This incubation resulted in the limited passive generation of small labeled species (Fig. 4A). Size-exclusion chromatography showed a slight decrease in the size of the macromolecular peak (K_{av}~0) with 91%, 87%, and 84% of the total CPMs accounted for at each timepoint, respectively. The small species peak (K_{av}~1) increased correspondingly with 8%, 12%, and 14% of the total CPMs at days 7, 14, and 21, respectively.

Next, ^{125}I-TGF-β1 was mixed with acellular peptide hydrogels prior to gelation and both bath and hydrogel samples were collected at days 7, 14, and 21. The ^{125}I macromolecular and small-species peaks released to the equilibration bath from these acellular peptide hydrogels were nearly equivalent at day 7, with 49% macromolecular compared to 36% small species (Fig. 4B). The percentage of small species in the bath increased with time, and at day 21 only 10% of the ^{125}I-species in the equilibration bath were macromolecular and 85% were small molecules. In contrast, the ^{125}I-species retained within the acellular peptide hydrogels was 96% macromolecular at day 7 and 80% macromolecular at day 21 (Fig. 4C), whereas the small-labeled species present within the acellular peptide were 2% and 11% at days 7 and 21, respectively.

When ^{125}I-TGF-β1 was mixed with BMSC-seeded peptide hydrogels, the relative abundance of small-labeled species in the medium at day 7 was higher than for acellular hydrogels (Figs. 4D vs. 4B). At day 7, macromolecular species accounted for only 23% of CPMs while small molecules accounted for 54% (compared to 49% and 36% for acellular, respectively, see above). By day 21, BMSC-seeded and acellular hydrogels showed comparable abundance of labeled species, with 6% macromolecular and 68% small molecule for BMSC-seeded hydrogels (compared to 10% and 85% for acellular, respectively, see above).

Peptide Hydrogels Deliver Chondrogenic Levels of TGF-β1 to Encapsulated BMSCs

To determine whether peptide adsorbed TGF-β1 could stimulate chondrogenesis of encapsulated bovine BMSCs, $(KLDL)_3$-peptide solution was mixed with either 10 ng/mL (Ads-TGF-10) or 100 ng/mL (Ads-TGF-100) unlabeled TGF-β1 immediately prior to cell encapsulation. DNA content was ~50% higher in Ads-TGF-100 peptide hydrogels than in either TGF-β1-free controls or medium-delivered TGF-β1 hydrogels at day 14 ($p<0.01$, Fig. 5A), while Ads-TGF-10 was not different from either group. sGAG content was equivalent for Ads-TGF-100 and medium-delivered TGF-β1 at day 7 and both were ~4-fold higher than TGF-β1-free controls ($p<0.001$, Fig. 5B). In contrast, Ads-TGF-10 did not stimulate sGAG accumulation compared to TGF-β1-free controls. By day 14, sGAG content for Ads-

TGF-100 peptide hydrogels was 5-fold higher than TGF-β1-free controls (Fig. 6B, $p<0.001$) and nearly 2-fold higher than medium-delivered TGF-β1 ($p<0.05$).

TGF-β1 Adsorbed Peptide Hydrogels Stimulate Chondrogenesis of Equine BMSCs

Foal equine BMSCs were encapsulated within TGF-β1 adsorbed peptide hydrogels to test whether this delivery technology could stimulate chondrogenesis in a species used as a translational in vivo model of cartilage repair [32,33]. Since Ads-TGF-100 was successful in the bovine pilot study, this concentration was chosen for the equine studies. Hydrogels were analyzed at days 7, 14, and 21. When foal equine BMSCs were encapsulated in Ads-TGF-100 peptide hydrogels and cultured in TGF-β1-free medium, DNA content was 50% higher than in TGF-β1-free controls by day 7 (Fig. 6A, $p<0.001$) and was statistically equivalent to hydrogels with TGF-β1 supplemented medium. No further increase in DNA content after day 7 was seen for Ads-TGF-100 stimulated BMSCs resulting in 60% lower DNA content than for medium-delivered TGF-β1 at day 21 ($p<0.001$). Ads-TGF-100 BMSCs accumulated comparable sGAG to medium-delivered TGF-β1 throughout the entire 21 day culture period (Fig. 6B) with a final sGAG content that was 25-fold higher than TGF-β1-free controls ($p<0.001$). Consistent with sGAG content, proteoglycan biosynthesis with adsorbed TGF-β1 stimulation was either higher or equivalent to hydrogels cultured in TGF-β1 supplemented medium through 21 days (Fig. 6C) and was 4-fold higher than the TGF-β1-free control at day 21 ($p<0.001$). In addition, the sGAG retained within the hydrogel as a percentage of the total produced (i.e. both sGAG retained as well as lost to the conditioned medium) was 65% and 60% for adsorbed and medium-delivered TGF-β1, respectively, at day 7 (difference was not significant), and remained constant over 21 days in culture (Fig. 6D).

The solid matrix as a percentage of the total wet mass for BMSC-seeded hydrogels with Ads-TGF-100 was equivalent to hydrogels in TGF-β1 supplemented medium at days 7 and 14 (Fig. 6E), while by day 21 Ads-TGF-100 BMSCs had produced 25% less solid matrix than hydrogels with medium-delivered TGF-β1 ($p<0.05$). However, Ads-TGF-100 BMSCs still had more than 2-fold higher percentage solid than TGF-β1-free controls ($p<0.001$). The protein biosynthesis rate for Ads-TGF-100 BMSCs was equivalent to medium-delivered TGF-β1 at day 7 (Fig. 6F), but dropped to 50% of medium-delivered TGF-β1 at days 14 and 21 ($p<0.001$). Ads-TGF-100 BMSCs had more than 2-fold higher protein biosynthesis than TGF-β1-free controls at day 21 ($p<0.01$).

Discussion

Delivery of TGF-β1 for in vivo applications requires controlled local release to ensure the desired, targeted effects of this potent growth factor[2]. Self-assembling peptide hydrogels are capable of retaining TGF-β1, maintaining its bioactivity, and stimulating chondrogenesis of encapsulated BMSCs over 21 days in culture. Uptake was only minimally reduced by 100-fold excess unlabeled TGF-β1 demonstrating the high loading capacity and potential dosing flexibility for these peptide hydrogels. Simple mixing with the peptide solution prior to assembly enabled efficient delivery to and stimulation of the encapsulated cells, resulting in degradation of the ligand and release of the radiolabel.

While TGF-β1 encapsulation in acellular peptide and agarose hydrogels resulted in TGF-β1 retention in both hydrogels for 21 days, uptake ratio experiments (Fig. 1) showed substantially higher TGF-β1 uptake for peptide than for agarose. Consistent results were seen in TGF-β1 release experiments (Fig. 2) with reduced initial and total release for peptide as compared to agarose, suggesting that the peptide was capable of sustaining TGF-β1 release at a slower rate for a longer period of time. These experiments demonstrate the capacity of TGF-β1 to adsorb to two very different biomaterials, in addition to an interaction

between TGF-β1 and $(KLDL)_3$ peptide that was not present with agarose. This is consistent with the observed stimulation of chondrogenesis by TGF-β1 when delivered by both peptide and agarose hydrogels[34].

There are likely multiple mechanisms driving TGF-β1 adsorption including electrostatic interactions associated with the peptide hydrogel polyampholytic composition as well as excluded volume effects associated with both peptide and agarose. TGF-β1 has been adsorbed to a wide range of materials including titanium fiber[35], collagen-coated and uncoated titanium alloys[36], acidic gelatin[9,10], and functionalized dextrose[13]. The capacity for TGF-β1 to adsorb to this diverse range of materials is consistent with our results showing TGF-β1 adsorption to both uncharged agarose hydrogels and to an even greater extent to amphiphilic, zwitterionic peptide hydrogels.

Given that the pore size of 2% low-melting-point agarose is ~200 nm[37] and in $(KLDL)_3$ peptide the average fiber to fiber spacing is ~370nm[38], while the dimensions of TGF-β1 are less than 6 nm[39], the higher TGF-β1 uptake in $(KLDL)_3$ peptide than agarose hydrogels is not likely to be due to restricted diffusion[40]. Rather, it may be related to the presence of negatively-charged aspartic acid residues in $(KLDL)_3$ peptide since basic TGF-β1 (pI=9.5)[10] has been shown to interact electrostatically with acidic gelatin[9,10], anionic functionalized dextran[13], and alginate sulfate[41]. In addition, electrostatic interactions determine the release of small dye molecules from self-assembling peptides[22,40], and these peptides have been shown to bind numerous growth factors including PDGF-BB, VEGF-A, bFGF, and angiopoietin-1 likely through non-covalent adsorption to the peptide nanofibers[20,40].

Acellular peptide hydrogels preferentially retained macromolecular ^{125}I-labeled species and lost small ^{125}I-labeled species to the bath (Figs. 4B vs. 4C). In order to determine whether the peptide hydrogel itself would cause TGF degradation and hence increased the amount of small labeled species in the bath, we can compare the amount of small ^{125}I-species released from acellular peptide hydrogels to the spontaneous dissociation of ^{125}I-TGF-β1 (Fig. 4A). It is important to note that the size exclusion chromatography shown in Fig. 4 characterized the molecular size distribution of the sample and that the area under the curve in Fig. 4 does not represent the absolute amount of a particular species in any condition. To estimate the quantity of small labeled species released from acellular peptide hydrogels at day 7, the fraction of small labeled species in Fig. 4B (42% from the area under the peak near K_{av}=1) can be multiplied with the amount released shown in Fig. 3B (2%/day at day 7 of the total loaded ^{125}I-TGF) to yield a result of ~0.8% small labeled species released on day 7. This is consistent with the spontaneous dissociation of ^{125}I-label from ^{125}I-TGF-β1 (8% small species cumulatively generated from day 0–7, Fig. 4A) and the substantially higher uptake ratio for ^{125}I-TGF-β1 than for free ^{125}I, which suggests that the small labeled species will diffuse out of the gels and accumulate in the bath (Fig. 1). Thus we conclude that small labeled species present in the bath of acellular peptide hydrogels accumulated by passive dissociation of the ^{125}I-label from TGF and diffusion out of the gel. Furthermore, the hydrogel did not cause or increase the degradation of TGF within acellular peptide hydrogels.

BMSC-seeded peptide hydrogels released nearly twice as much ^{125}I-labeled species as acellular hydrogels (Fig. 3C). Size exclusion chromatography analysis showed that BMSC-seeded peptide hydrogels also preferentially released small radiolabeled species, in an equal or greater proportion than for the acellular gels (Figs. 4B vs. 4D, Day 7). Taken together, these results suggest that the additional release of small ^{125}I-labeled species from BMSC-seeded peptide hydrogels is the result of active degradation of TGF-β1 by the encapsulated cells. This is consistent with radiolabel analyses performed previously for several ligands which show cellular internalization and breakdown of TGF-β1[42,43], epidermal growth

factor[44], and tumor necrosis factor[45]. In addition, these results are consistent with a recent study of fibrin hydrogel delivered TGF-β1[12] which showed that cell-seeded hydrogels dramatically reduced the release of immunoreactive TGF-β1 (as detected by ELISA) as compared to acellular gels.

Cellular ligand internalization and active degradation, leading to depletion of the ligand concentration at the cell surface, is a mechanism consistent with a recent model showing the importance of ligand depletion in dose dependent modulation of TGF-β1 signaling[34,43]. It is therefore possible that self-assembling peptides may enable control over the encapsulated cell phenotype by controlling the duration of exposure to TGF-β1.

TGF-β1 uptake in peptide hydrogels depends on whether it is adsorbed prior to peptide assembly or allowed to diffuse into assembled hydrogels (Fig. 1). A potential explanation is that when TGF-β1 (< 6 nm[39]) is added to peptide solution prior to assembly, it gets entrapped within assembling nanofibers (~30nm diameter[38]) which is not possible when TGF-β1 is allowed to diffuse into previously assembled peptide. Such a mechanism may suggest that there is a population of TGF-β1 molecules that is inaccessible to encapsulated cells due to steric constraint within the nanofibers. This is consistent with the release per day for BMSC-seeded peptide hydrogels (Fig. 3B), which is much higher from days 0–8 and reaches a low steady level from days 11–21, potentially because all cell accessible TGF-β1 was consumed and released during days 0–8. Phosphorylation of Smad 2/3 by the TGF-β1 receptor was not able to be detected after day 7, consistent with this explanation. In addition, since as little as 4 days of TGF-β1 stimulation upregulated proteoglycan synthesis at day 21, the TGF-β1 delivered by peptide hydrogels is likely sufficient to ensure progenitor cell commitment to chondrogenesis[34].

It is well established that chondrogenesis can be stimulated by continuously supplementing culture medium with TGF-β1 and refreshing the medium and TGF-β1 dose every 2–3 days[3,5,46]. In this study, with 750 μL of medium added to 50 μL hydrogels, the total quantity of medium-delivered TGF-β1 added during 21 days of culture (assuming 9 medium changes and 10 ng/mL TGF-β1) is more than 67 ng. In contrast, the total dose of TGF-β1 adsorbed to peptide hydrogels for chondrogenesis (100 ng/mL in a 50 μL gel for Ads-TGF-100) was 5 ng, over an order of magnitude lower. Nonetheless, 5ng of adsorbed TGF-β1 stimulated comparable sGAG content and proteoglycan synthesis to medium-delivered TGF-β1 (Fig. 6) consistent with other recent reports[47]. Thus, self-assembling peptide hydrogels can be utilized to locally target a relatively small quantity of growth factor to encapsulated cells and produce a nearly equivalent chondrogenic outcome, demonstrating the efficiency and efficacy of this system. This is especially important in cartilage repair procedures since uncontrolled doses of TGF-β1 into the joint cavity *in vivo* can cause an inflammatory fibrotic response in multiple tissues[48].

Conclusion

Self-assembling peptide hydrogels delivered bioactive TGF-β1 at a dose that stimulated comparable chondrogenesis to medium-delivered TGF-β1, while utilizing over an order of magnitude less growth factor. Peptide hydrogels had significantly higher TGF-β1 uptake and retained significantly more TGF-β1 during release experiments than agarose hydrogels. Introducing unlabeled TGF-β1 at two orders of magnitude higher concentration than radiolabeled TGF-β1 had a minor impact on uptake. Coupled with the easily synthesized 8–16 residue structure of the peptides, their capacity to deliver encapsulated cells, and their ability to fill irregularly shaped defects, these results demonstrate that self-assembling peptide hydrogels are a versatile controlled release platform suitable for testing in animal models of cartilage defect repair.

Acknowledgments

The authors would like to thank Dr. Richard T. Lee for numerous discussions and invaluable advice on strategies to tether and adsorb growth factors to self-assembling peptide hydrogels. This work was funded by the National Institutes of Health (NIH EB003805 and NIH AR60331), a National Institutes of Health Molecular, Cell, and Tissue Biomechanics Training Grant Fellowship (P.W.K.), and an Arthritis Foundation Postdoctoral Fellowship (E.J.V.).

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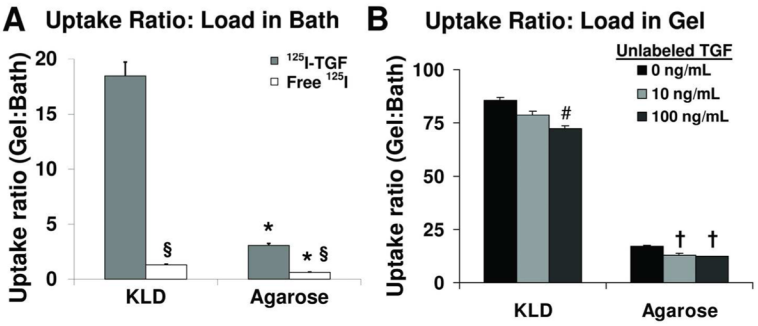

Figure 1. TGF-β1 Uptake Ratio in acellular KLD Peptide and Agarose Hydrogels

(A) Uptake ratio of ^{125}I-TGF-β1 when added to the equilibration bath after gelation. Uptake ratio of free ^{125}I label added to the bath after gelation shown for comparison. Unlabeled TGF-β1 was not added for experiments in (A). mean ± SEM; n=6; § vs. ^{125}I-TGF; * vs. KLD; $p<0.001$. (B) Uptake ratio of ^{125}I-TGF-β1 when added to the hydrogel solution prior to gelation simultaneously with 0, 10, or 100 ng/mL of unlabeled TGF-β1. mean ± SEM; n=4; # vs. 0 ng/mL in KLD; † vs. 0 ng/mL in agarose; $p<0.05$.

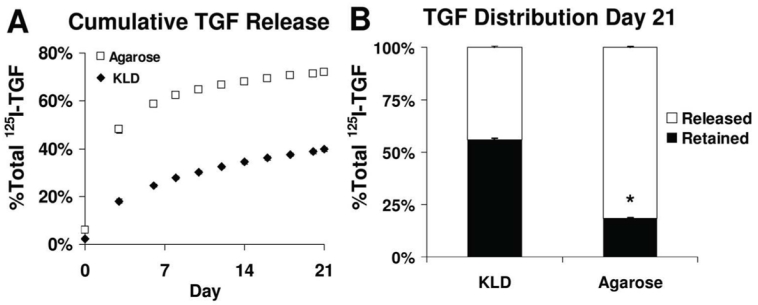

Figure 2. Acellular KLD peptide hydrogels retain higher TGF-β1 than agarose hydrogels

^{125}I-TGF-β1 was adsorbed to the hydrogel prior to gelation for all experiments. Unlabeled TGF-β1 was not added. (A) Cumulative release of ^{125}I-TGF-β1 to the bath. (B) Total ^{125}I-TGF-β1 released to the bath (Released) and retained in the hydrogel (Retained) at day 21. mean ± SEM; n=6; * vs. KLD; $p<0.05$.

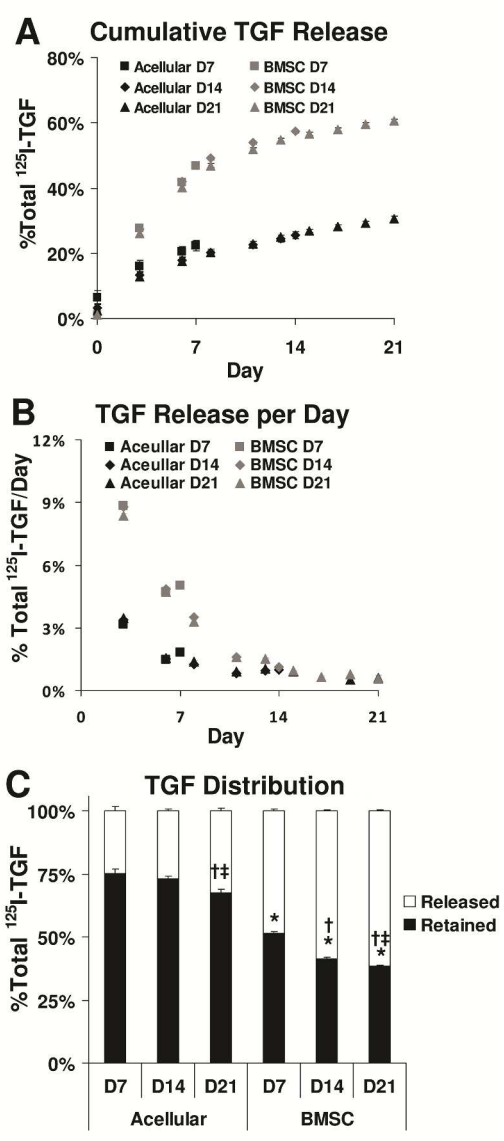

Figure 3. BMSC-encapsulation increases TGF-β1 release from KLD peptide hydrogels

Both ^{125}I-TGF-β1 (1.4 ng/mL) and unlabeled TGF-β1 (100 ng/mL) were adsorbed to the hydrogel prior to gelation for all experiments. (A) Cumulative release and (B) release per day to the bath of ^{125}I-TGF-β1 for experiments terminated at 7, 14, & 21 days (D7, D14, & D21). (C) Total TGF-β1 released to the bath (Released) and retained in the hydrogel (Retained) at day 7, 14, & 21. mean ± SEM; n=4; † vs. day 7; ‡ vs. day 14; * vs. acellular; $p<0.05$.

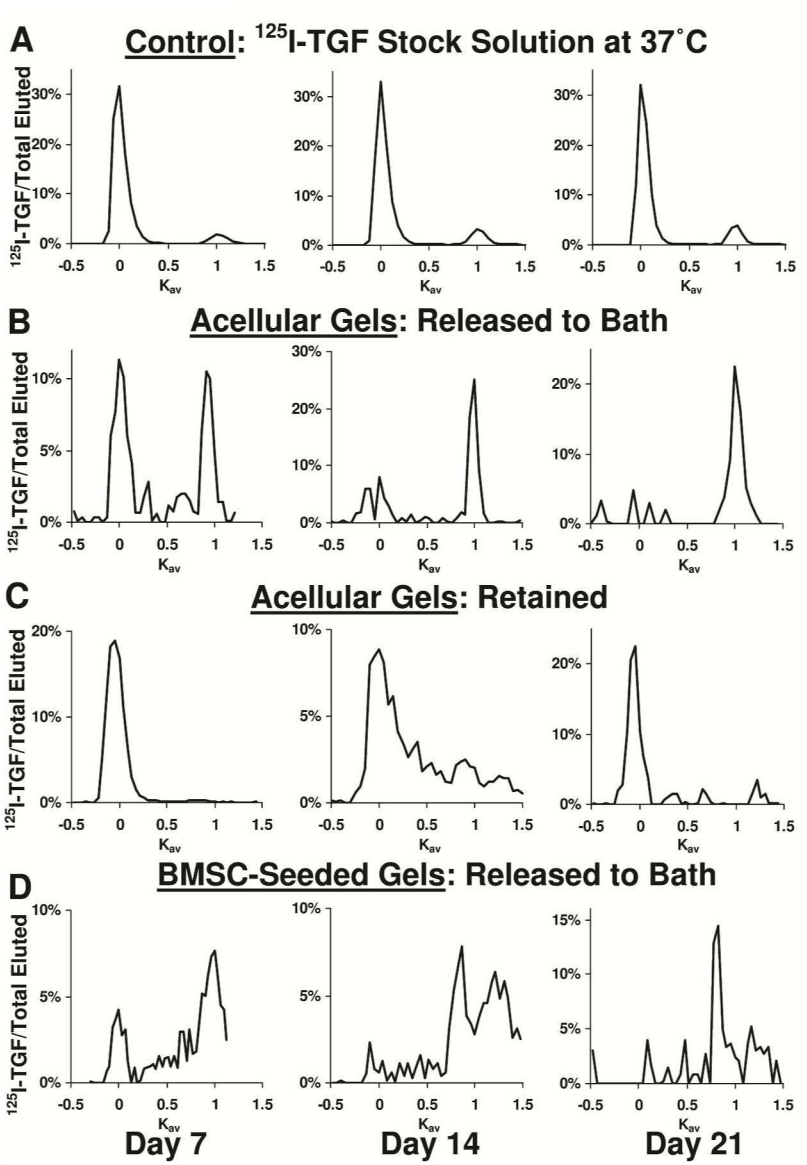

Figure 4. Size exculsion chromatography of ^{125}I-labeled species by Sephadex G25

(A) Control samples of ^{125}I-TGF-β1 stock solution after 7, 14, & 21 days of incubation at 37°C. (B–D) ^{125}I-TGF-β1 (1.4 ng/mL) plus unlabeled TGF-β1 (100 ng/mL) was adsorbed to KLD peptide hydrogels. (B) ^{125}I-TGF-β1 released to the bath from acellular peptide hydrogels. (C) ^{125}I-TGF-β1 retained in acellular peptide hydrogels. (D) ^{125}I-TGF-β1 released to the bath from bovine BMSC-seeded peptide hydrogels.

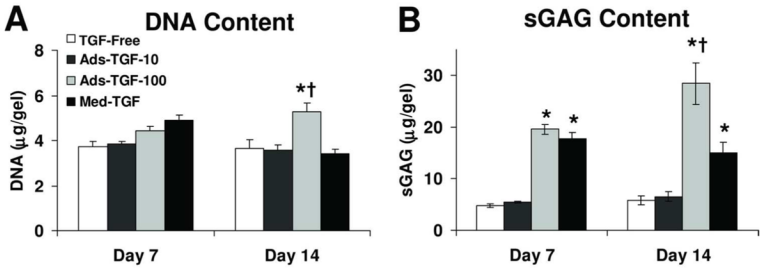

Figure 5. Adsorbed TGF-β1 stimulates chondrogenesis of bovine BMSCs encapsulated in KLD peptide hydrogels

TGF-β1 was adsorbed to KLD peptide prior to gelation at 10 ng/mL (Ads-TGF-10) or 100 ng/mL (Ads-TGF-100). Control hydrogels were cultured in either TGF-β1-free medium (TGF-Free) or with medium containing 10 ng/mL TGF-β1 (Med-TGF). (A) DNA content. (B) sGAG content. mean ± SEM; n=4; * vs. TGF-Free; † vs. Med-TGF; $p<0.05$.

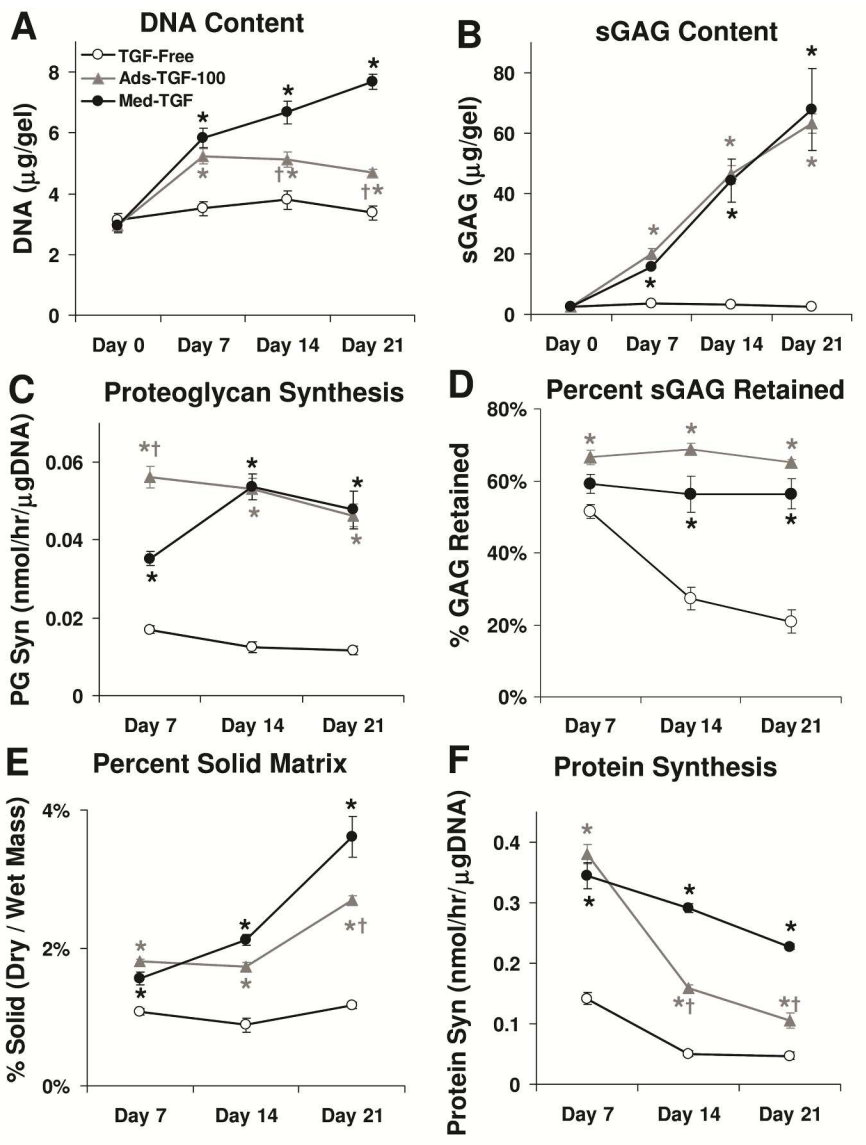

Figure 6. Adsorbed TGF-β1 stimulates chondrogenesis of young equine BMSCs encapsulated in KLD peptide hydrogels

TGF-β1 was adsorbed to KLD peptide prior to gelation at 100 ng/mL (Ads-TGF-100). Control hydrogels were cultured in either TGF-β1-free medium (TGF-Free) or with medium containing 10 ng/mL TGF-β1 (Med-TGF). (A) DNA content. (B) sGAG content. (C) Proteoglycan synthesis. (D) Percent sGAG retained within the hydrogel. (E) Hydrogel percent solid content. (F) Protein synthesis. mean ± SEM; n=8 (4 gels x 2 horses); * vs. TGF-Free; † vs. Med-TGF; $p<0.05$.