Aggregate-Inducing Biomaterials for Regeneration of the Ligament-Bone Insertion

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Introduction: For ligament reconstruction, a secure interface between the ligament graft and bone is essential for proper function. Native ligament-bone insertion points contain a region of fibrocartilage with a gradual transition of mechanical properties. Aggrecan, an extracellular matrix (ECM) proteoglycan found in cartilage and ligament-bone insertion points, has been shown to promote aggregation and production of cartilaginous ECM by dermal fibroblasts (French, MM. Ann Biomed Eng. 2004;32:50-56). Our laboratory is investigating a novel hydrogel system of oligo(poly(ethylene glycol) fumarate) (OPF) as a cell carrier for regeneration of ligament tissue. To further investigate the role of aggrecan and cellular aggregation in differentiation down the (fibro)chondrocytic lineage, we explored the effect of aggrecan-coated and nonadhesive surfaces on the morphology and gene expression of medial collateral ligament (MCL) fibroblasts and marrow stromal cells (MSCs) and examined aggrecan retention in OPF hydrogels over 4 weeks *in vitro*.

Methods: Wells of a 24-well plate were treated with 5 µg of bovine aggrecan by suspending aggrecan in saline and allowing it to evaporate. Bovine MCL fibroblasts (P1) and MSCs (P2) were plated at $8.8x10^4$ cells/well on aggrecan-coated surfaces, nonadhesive surfaces that resist cell attachment, and control surfaces in media with fetal bovine serum. Cells were also cultured in serum-free media on aggrecan and control surfaces, and 5 µg of aggrecan were added to the media on control surfaces during seeding or 24 hrs after. Morphology was observed over 5 days. Gene expression on all surfaces after 3 days (with serum) was analyzed by reverse transcription-polymerase chain reaction (RT-PCR) for collagen I, collagen II, aggrecan, and peroxisome proliferator-activated receptor γ2 (PPAR-γ2). On aggrecan surfaces, all cells in a well were analyzed by RT-PCR, and aggregates were also analyzed separately from non-aggregating cells. To study aggrecan-retention in hydrogels, aggrecan was noncovalently incorporated into OPF/PEG-diacrylate (50:50 by weight) gels at 0, 150, and 300 µg/ml. OPF 3K and 10K (synthesized from 3.4 and 10 kDa poly(ethylene glycol), respectively), which have different swelling properties, were used. All hydrogels were swelled in saline at 37°C. Aggrecan content remaining in the hydrogels over 28 days was determined via proteinase K digestion and dimethylmethylene blue (DMMB) assay, compared to a standard curve.

Results: Aggrecan-coated surfaces induced formation of cell aggregates within 24 hrs in both cell types. Aggregate formation was not dependent of the presence of serum proteins, and suspended aggrecan did not induce aggregation, indicating that aggrecan must be adsorbed to the surface. Nonadherent aggregates also formed on nonadhesive surfaces for both cell types. RT-PCR results suggest that aggregating cells on aggrecan and nonadhesive surfaces upregulate gene expression of aggrecan, compared to control surfaces (Figure 1). MCL fibroblasts and MSCs on aggrecan surfaces signifcantly upregulated aggrecan expression with 3.10±0.22 and 2.08±0.54 fold regulation, respectively, and aggregate-only samples experienced larger 5.40±1.15 and 6.84±0.36 fold regulation, respectively. Similarly, nonadhesive MCL aggregates experienced a 7.02±0.67 fold upregulation of aggrecan, while MSCs experienced a larger regulation of 64.01±6.93 fold. The DMMB assay demonstrated that aggrecan was completely released from 10K, 150 µg/ml hydrogels by day 14, and aggrecan was released by day 21 from 10K, 300 µg/ml gels (Figure 2). In 3K, 300 µg/ml hydrogels, aggrecan was retained until day 28, demonstrating that release profiles are altered by the OPF swelling properties and initial loading concentrations, with greater swelling permitting earlier release from gels.

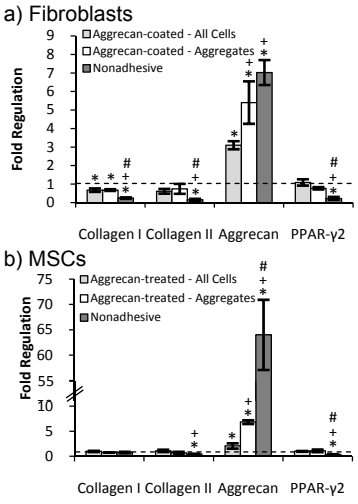

Figure 1. Gene expression of (a) MCL fibroblasts and (b) MSCs on aggrecan-coated and nonadhesive surfaces, compared to control surfaces. Fibroblast and MSC aggregates upregulated aggrecan gene expression on aggrecan-coated and nonadhesive surfaces (n=3). * indicates significantly different from controls ($p<0.05$). + indicates significantly different from all-cell samples ($p<0.05$). # indicates significantly different from aggregate only samples ($p<0.05$). Note the different scales of the axes in a) and b).

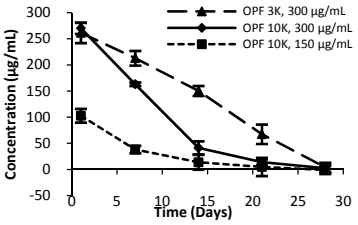

Figure 2. *In vitro* release of aggrecan from OPF 3K and 10K hydrogels, as measured by dimethylmethylene blue (DMMB) assay over 28 days, with initial starting concentrations of 150 and 300 µg/mL. (n=4)

Conclusions: These results indicate that aggregation of MCL fibroblasts and MSCs upregulates gene expression of a key cartilaginous ECM protein, aggrecan, in fibroblasts and MSCs after 3 days. These results also present a novel method to control aggrecan exposure in a 3D hydrophilic environment to study the effect on aggregation and ECM production for regeneration of the ligament-bone insertion. In the future, similar aggregate-inducing or aggrecan-containing treatments may be utilized in combination with a variety of biomaterial scaffolds to promote chondrogenic/fibrochondrogenic differentiation at the ligament-bone insertion point.

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