Hydrogels for vocal fold tissue engineering and repair

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INTRODUCTION

The human vocal folds are paired structures roughly 10-15 mm in length and 3-5mm thick, which are brought into apposition across the airway for sound production. Each vocal fold is a laminated structure consisting of pliable vibratory layer of connective tissue, known as the lamina propria (LP), sandwiched between epithelium and muscle. The human LP is often further subdivided into the superficial (SLP), intermediate (ILP), and deep (DLP) layers [1]. The SLP is believed to be the region of the vocal fold that sustains the most stress during vibration [2] and, thus, is particularly prone to scarring from mechanical damage from voice abuse or overuse [3]. Scarred SLP can also result from benign and malignant disease processes requiring ablative surgery or radiation therapy, or from prolonged endotracheal intubation [4]. The altered geometry and increased tissue stiffness associated with vocal fold scarring cause voice changes ranging from hoarseness to complete voice loss. Voice loss or chronic voice impairment secondary to vocal fold scarring is a debilitating disorder estimated to affect 2-6 million people in the US alone. Since little can currently be done to treat vocal fold scarring, alternate treatment methods are needed. One such alternate approach is the use of an implant to restore normal physiology.

The vocal fold SLP is a highly hydrated loose, relatively homogenous connective tissue layer composed of low concentrations of collagen and elastin relative to connective tissues such as dermis [5, 6]. Several materials have been examined as possible replacements for the SLP, including collagen [7], fat [8], and hylan b [9], a derivative of hyaluronan (HA). Although each of these implants has resulted in some degree of improvement in vocal fold function, gel resorption and/or contraction have limited the general success of these materials.

In this study, three biomaterials or biomaterial composites were investigated for SLP tissue restoration, with a focus on minimizing construct volume loss over time. In this study, collagen was combined with either HA or alginate in an effort to inhibit fibroblast mediated contraction of the hydrogels. In addition, the behavior of vocal fold fibroblasts in hydrogels of the spontaneously self-assembling peptide RAD-16-II, a member of a novel class of synthetic biomaterials which are being actively pursued as scaffolds for tissue repair, was also investigated.

EXPERIMENTAL

Cell isolation

Primary vocal fold fibroblasts were isolated from the midmembranous LP of 6-12 month old pigs (MIT, Division of Comparative Medicine) by primary explant. Explant cultures were maintained at 37^{o}C / 5% CO_2 in Fibroblast Growth Media-2 (FBM-2, Cambrex) supplemented with 100 U/mL penicillin/streptomycin (P/S) and 0.25μg/mL fungizone (Gibco) in place of the gentamycin supplied with the FBM-2 media bullet kit. After one week, fibroblasts that had migrated out of the tissue were passaged and maintained in FBM-2 with P/S but without fungizone. All hydrogel constructs were similarly grown in FBM-2 supplemented with P/S but not with fungizone. Animal tissue was obtained with the approval and according to the guidelines of the MIT animal care committee.

Collagen-HA and collagen-alginate, in vitro assays

An ice-cold 2% HA solution, prepared by sterile filtration, was added to neutralized ice-cold rat tail collagen (BD Biosciences) such that the final concentrations of collagen and HA prior to cell encapsulation were each approximately 5mg/mL. Alginate from Laminaria hyperborean (gift of FMC BioPolymer (Philadelphia, PA, USA)) was used to prepare a 2% alginate solution, which was sterilized by autoclaving. A collagen-alginate gel solution was prepared using the same methodology as that for the collagen-HA gel solution.

Vocal fold fibroblasts at passage 6 were detached with trypsin-EDTA (Gibco-BRL) and resuspended in DMEM without FBS. 250uL of the cell suspension was added to 5.4mL of each gel mixture, such that the final concentration of cells in the gels was $1x10^6$ cells/mL. Cell number was determined using a hemacytometer and cell viability immediately prior to encapsulation was greater than 95%, as determined by trypan blue exclusion. The gel solutions were then aliquoted in 100uL amounts into Millicell CM culture inserts (12mm, 0.4 μm pore size). In the case of collagen-alginate gels, the inserts were placed under constant stirring conditions in a bath 75 mM $CaCl_2$ for 15 minutes, after which fresh media was added to each construct. Collagen-HA composites were gelled at 37°C in an incubator for 90 minutes, after which media was added. Constructs were cultured under static conditions for 4 wks in vitro at 37^oC / 5% CO_2. Media was changed every two days. Constructs were analyzed histologically and biochemically at days 7, 14, and 28.

RAD-16-II and collagen-alginate based gels, in vitro culture

Based on results of the 4 wk trials of collagen-alginate and collagen-HA hydrogels, a collagen-alginate based gel was selected as a control gel for in vitro trials of synthetic, self-assembling peptide RAD-16-II. Specifically, the peptide RAD-16-II with sequence AcN-$(RARADADA)_2$-CNH_2 (SynPep Corp.) was dissolved in tissue culture grade water to a concentration of 10mg/mL and sonicated for 20 minutes prior to use. Isolated vocal fold fibroblasts at passage 6 were resuspended at $2x10^6$ cells/mL in 20% sucrose. Equal volumes of peptide solution and cell suspension were then mixed, and 150μL of the cell-peptide suspension was pipetted into 12 mm CM inserts (Millipore). The upper and lower surfaces of the gel were then exposed to FBM-2 media to initiate self-assembly of the peptide. The gels were transferred to 37^oC / 5% CO_2 for 1 hr, after which media was exchanged. Cells were similarly encapsulated into a collagen-alginate based gel, using the methodology described in the previous section. Constructs were maintained at 37^oC / 5% CO_2 with media changes every two days. Time points for the RAD-16-II gels were collected at time points 7, 14, 21. For the collagen-alginate based control gels an additional time point was taken at week 6.

Biochemical analyses

Total collagen, sulfated GAG and DNA

Six gels per time point were harvested for biochemical analyses and their wet weights were recorded. Gels were stored at -80^oC until time of analysis. At time of analysis, disks were solubilized using 25μg/mL proteinase K in 55 mM sodium citrate, 0.9% NaCl buffer overnight at

55°C. Hydroxyproline (OHPr), sulfated GAG, and DNA levels were measured for each construct. OHPr was determined spectrophotometrically from after acid hydrolysis (6N HCl at 115^{o}C overnight) of sample digest aliquots and subsequent reaction with chloramine T and p-dimethylbenzaldehyde [10]. DNA content was measured relative to calf thymus DNA standards (Sigma) using the picogreen assay reagents (Molecular Probes). DNA was converted to cell number using the conversion factor for pig DNA of 6.6×10^{-12} pg DNA per cell [11]. Total sulfated GAG was measured using a modified DMMB assay [12] and chondroitin 4-sulfate standards (Accurate Chemical and Scientific, Corp).

Histological and immunohistochemical analysis

Cell morphology

At each time point, 2-3 gels were fixed in 4% paraformaldehyde for 20 min for histological analyses. These gels were incubated in 160nM rhodamine phalloidin (Molecular Probes), a high-affinity probe for F-actin, in PBS at 4^{o}C overnight.

Cell proliferation and ECM production

An additional 2-3 gels were fixed in 4% paraformaldehyde in PBS at RT, washed with PBS, and were transferred to OCT media overnight at 4°C and snap frozen. 20μm cryosections transverse to the diameter of the cylindrical disk were cut.

GAG and collagen

Sections were histologically stained for sulfated GAG and total collagen. OCT media was removed in running dH_2O, and GAG was detected by exposing sections to toluidine blue solution [13]. Collagen was analyzed using a modified Gomori's trichrome stain (Biocare Medical), according to manufacture's instructions. To highlight newly synthesized collagen, reticular fibers were stained using the Gordon & Sweet's reticular collagen staining kit (NewComer Supply). according to manufacturer's instructions.

RESULTS

Hydrogel contraction and mass

Changes in average gel mass are depicted in Figure 1. Generally, a decrease in construct mass with time can be primarily attributed to loss in gel volume or water content. For collagen-HA and RAD-16-II gels, contraction of gel diameter with time was observed, in particular for the collagen-HA gels. For collagen-alginate gels, no reduction in gel diameter was noted for up to six weeks in culture. The collagen-HA gels showed the most significant decrease in mass with time, although collagen-alginate and RAD-16-II gels appeared to maintain their weights.

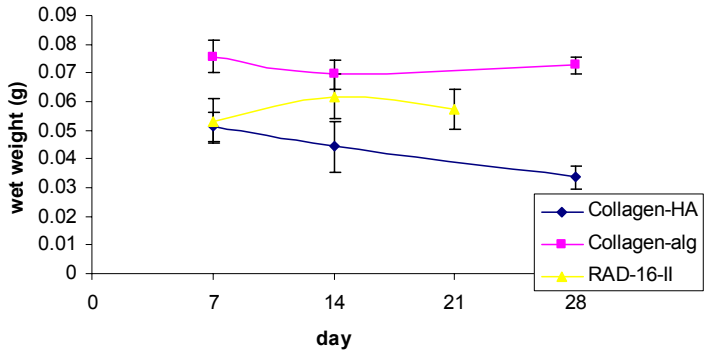

Figure 1: construct mass with time in culture

Fibroblast Appearance and Proliferation

Cell number in each gel remained fairly constant with time in culture (Figure 2). However, cell morphology and cell interconnections varied with gel type. In collagen-HA scaffolds, cells were elongated and primarily spindle shaped. Interconnections generally occurred at the spindle tips of the cells. In alginate cultures, cells had bulbous bodies with thin "tails". Generally, cells were not interconnected. However, sometimes, cells had divided without detaching from each other, creating long linear cell groups. Within the RAD-16-II gels, cells were homogeneously seeded within the gels at the time of encapsulation, as verified by microscopic examination of time zero gels. By the day 7 time point, cell clusters connected by numerous lamellopodia, were evident. These clusters of interdigitating, stellate-like cells were typical of observed cellular organization within the gels during the remaining culture period.

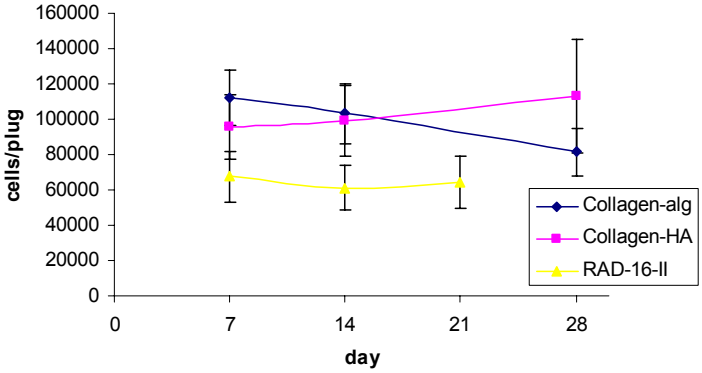

Figure 2: cellularity with time in culture

Production of ECM Constituents

Over the course of culture, the constructs were assessed for total sulfated GAG, OHPr, and DNA content. The OHPr content of the collagen-HA and the collagen-alginate gels decreased with time from wk 1 to wk 4 in a similar manner (Figure 3). However, when examined histologically, collagen synthesis did appear to be occurring in the collagen-alginate gels. Specifically, reticular collagen staining of the collagen-alginate gels indicated collagen

synthesis pericellularly and at the gel surfaces. However, similar signs of collagen synthesis were not noted for the collagen-HA gels. The loss of collagen from the collagen-alginate composites during the first several weeks of culture time seems to imply that the rate of collagen erosion from the gel initially exceeds the rate of new collagen production. However, when alginate cultures were continued to wk 6, an increase in OHPr to a level approximately 15% greater than that of culture day 7 was noted, indicating collagen accumulation with increased time in culture. For RAD-16-II gels, OHPr levels showed a general increase over the first several weeks in culture.

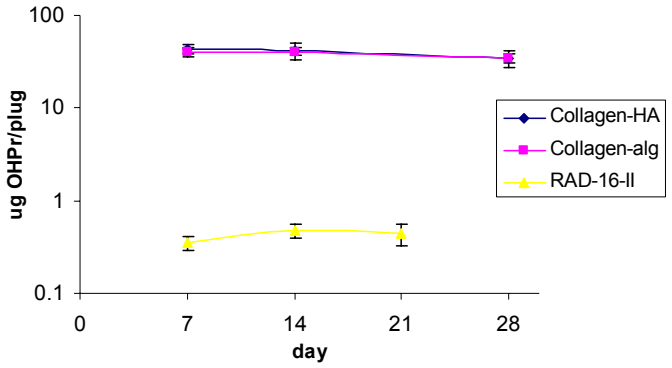

Figure 3: OHPr with time in culture

Histological and biochemical results (Figure 4) indicate a general increase in sulfated GAG levels in RAD-16-II gels with time. Also, toluidine blue indicated GAG production in the collagen-alginate, both pericellularly and at gel borders. Additionally, the density of GAG staining within the collagen-alginate gel center increased with time. However, little evidence for GAG synthesis was evident from the collagen-HA gel toluidine blue staining results.

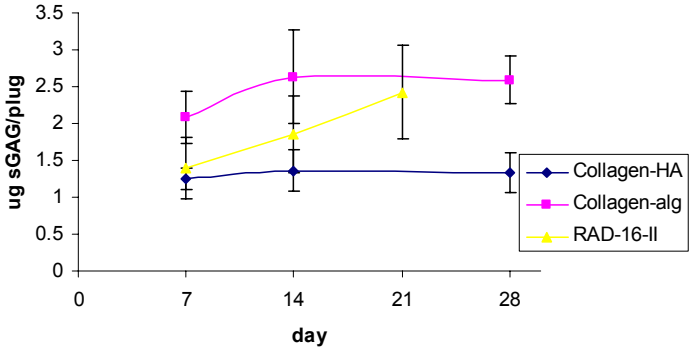

Figure 4: sulfated GAG with time in culture

CONCLUSIONS

Based on combined biochemical and histological evaluation, collagen-HA gels do not appear to promote new ECM synthesis by vocal fold fibroblasts. This is in contrast to collagen-

alginate gels, in which clear signs of matrix synthesis were evident. Apparently the presence of HA seems to inhibit new ECM production, whereas the presence of alginate does not appear to similarly inhibit matrix production. In addition, the collagen-alginate gel seems to support new matrix production without loss of gel mass with time or decrease in gel diameter, features that are desirable for vocal fold tissue engineering. The RAD-16-II gel similarly seems to support new ECM production with minimal gel contraction observed over the time period of study.

This work demonstrates the potential of self-assembling peptide RAD-16-II and collagen-alginate composites to maintain vocal fold fibroblasts and stimulate the synthesis and accumulation of ECM in 3D cell culture with minimal matrix contraction. Alginate has been studied extensively as a tissue engineering matrix for cartilage and for soft tissue augmentation. The peptide RAD-16-II used in this study represents one of a class of self-assembling peptides developed through molecular engineering. These peptides assemble into well-ordered nano-fibers with inter-fiber spacing of ~50-200nm, creating a nanoscale hydrogel network around encapsulated cells [14-16]. Since self-assemblying peptides can be modified to contain biologically-active motifs, the nanoscale cell-scaffold architecture may offer unique advantages for controlled peptide-cell signaling and cell-mediated peptide degradability. ECM synthesis in both of these matrices may be further stimulated by addition of diverse growth factors or by culture in bioreactors that increase nutrient transport or apply mechanical loads.

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