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Influence of glycosaminoglycan identity on vocal fold fibroblast behavior

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Abstract

Poly(ethylene glycol) (PEG) hydrogels have recently begun to be explored for the treatment of scarred vocal fold lamina propria due, in part, to their tunable mechanical properties, resistance to fibroblast-mediated contraction, and ability to be polymerized in situ. However, pure PEG gels lack intrinsic biochemical signals to guide cell behavior and generally fail to mimic the frequency-dependent viscoelastic response critical to normal superficial lamina propria function. Recent results suggest that incorporation of viscoelastic, bioactive substances, such as glycosaminoglycans (GAGs), into PEG networks may allow these gels to more closely approach the mechanical responses of normal vocal fold lamina propria while also stimulating desired vocal fold fibroblast behaviors. Although a number of vocal fold studies have examined the influence of hyaluronan (HA) on implant mechanics and vocal fold fibroblast responses, the effects of remaining GAG types have been relatively unexplored. This is significant since recent studies suggest that chondroitin sulfate C (CSC) and heparan sulfate (HS) are substantially altered in lamina propria scar. The present study was therefore designed to evaluate the effects of CSC and HS incorporation on PEG gel mechanical response and vocal fold fibroblast behavior relative to HA. As with PEG-HA, the viscoelasticity of PEG-CSC and PEG-HS gels more closely approached that of the normal vocal fold lamina propria than pure PEG hydrogels. In addition, collagen I deposition and fibronectin production were significantly higher in CSC than in HA gels, and levels myofibroblast marker SM- α -actin were greater in CSC and HS gels than in HA gels. Since collagen I, fibronectin, and SM- α -actin are generally elevated in lamina propria scar, these results suggest that CSC and HS may be undesirable for vocal fold implants relative to HA. Investigation of various signaling intermediates indicated that alterations in NF κ B-p50, NF κ B-p65, or pERK1/2 levels may underlie observed differences among the PEG-GAG gels.

INTRODUCTION

Voice disorders resulting from scarring of the vocal fold lamina propria can be debilitating in terms of quality of life. The vocal folds are paired, multi-layered structures, each consisting of an underlying skeletal muscle layer, followed by the lamina propria and

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overlying epithelium. The lamina propria is generally subdivided into the superficial lamina propria (commonly referred to as the SLP) and the vocal ligament [1]. During vocal fold vibration, the SLP is believed to move freely over the elastin- and collagen-rich vocal ligament, undergoing the high frequency and strain excursions required for cyclic vocal fold closure. When SLP pliability and physical volume are reduced by the abnormal extracellular matrix (ECM) deposition and fibroblast-mediated contracture associated with scarring, voice changes ranging from hoarseness to complete voice loss result, depending on the severity of the scar [2–3].

Vocal fold scar has proven difficult to treat with current surgical techniques and standard augmentation substances, such as collagen and fat [2, 4]. Researchers are therefore actively exploring alternative treatment routes, including the development of designer implants for functional SLP regeneration [5–12]. Poly(ethylene glycol) (PEG)-based hydrogels have recently begun to be examined as vocal fold augmentation substances [9, 11, 13–16] due, in part, to their tailorable mechanical properties, resistance to fibroblast-mediated contraction, and ability to be polymerized in situ. However, pure PEG hydrogels lack intrinsic biochemical signals to guide cell behavior and generally fail to mimic the frequency-dependent viscoelastic response critical to normal SLP function [9, 13–15, 17].

Recent results suggest that conjugating viscoelastic, bioactive substances, such as glycosaminoglycans (GAGs), into PEG networks may allow these hydrogels to more closely approach the mechanical responses of normal SLP while stimulating desired vocal fold fibroblast behaviors [13–14, 18]. GAGs are a family of charged polysaccharides which play key roles in inflammation, cell migration, and cell phenotype modulation [19] and which include unsulfated forms (e.g. hyaluronan (HA)) as well as sulfated forms (e.g. heparan sulfate (HS), chondroitin sulfate C (CSC), dermatan sulfate (DS), and keratan sulfate (KS)). While a number of vocal fold studies have examined the impact of HA addition on implant mechanics and/or fibroblast behaviors [6–7, 12–14, 18, 20] the influence of sulfated GAGs has been relatively unexplored. The present study was therefore designed to compare the effects of CSC and HS incorporation on PEG hydrogel biomechanical response and on associated vocal fold fibroblast behavior relative to HA. CSC and HS were selected for investigation over other sulfated GAGs for several reasons. First, although the DS-bearing proteoglycan decorin has been a focus of several vocal fold studies [21–22] due to its known role in dermal scar formation [23], recent quantitative measures suggest that expression of CSC, rather than DS, is substantially decreased in lamina propria scar [24]. In addition, a separate study has shown that levels of specific HS proteoglycans are altered in scarred lamina propria [25].

MATERIAL AND METHODS

Polymer Synthesis and Characterization

PEG-Diacrylate Synthesis—Diacrylate-derivatized PEG (PEGDA) was prepared as previously described by combining 0.1 mmol/ml dry PEG (10 kDa, Fluka), 0.4 mmol/ml acryloyl chloride, and 0.2 mmol/ml triethylamine in anhydrous dichloromethane and stirring under argon overnight [26]. The resulting solution was washed with 2 M K₂CO₃ and separated into aqueous and dichloromethane phases to remove HCl. The dichloromethane phase was subsequently dried with anhydrous MgSO₄, and PEGDA was precipitated in diethyl ether, filtered, and dried under vacuum. The extent of PEG diacrylation was determined by ¹H-NMR to be ~85%.

Synthesis of Methacrylate-Derivatized CSC and HS—In order to conjugate specific GAGs within the PEGDA-based hydrogel networks, each GAG type was methacrylate-derivatized according to standard protocols [27]. CSC (51 kDa, Sigma) and heparin (17 kDa,

Sigma) were dissolved in deionized water to achieve a 1 wt% final concentration, and the pH of each solution was adjusted to 8.0. A 10-fold molar excess of methacrylic anhydride (Polysciences) was added per disaccharide unit. Each reaction was allowed to proceed under constant stirring at 4 °C, with the solution pH being maintained at ~8.0 by periodic addition of 5 M NaOH. The reaction products (CSC-MA and HS-MA) were precipitated twice in chilled 95% ethanol, dialyzed against deionized water for 48 h, and lyophilized. The extent of methacrylate-derivatization was characterized by ¹H-NMR to be ~1–3% (per available –OH groups). These levels of methacrylate-derivatization have previously been shown to negligibly alter observed cell-GAG interactions [18, 27–29] yet to be sufficient to avoid significant sol [30].

Synthesis of Methacrylate-Derivatized Intermediate Molecular Weight HA—

Since the average molecular weight of HA in normal human vocal fold lamina propria is currently unknown, an intermediate molecular weight HA (~400 kDa) was selected for the present study [18]. To generate HA appropriate for the examination, high molecular weight HA (~1.65×10³ kDa, *Streptococcus equi*; Fluka) was first methacrylate-derivatized as above. The purified product was then dissolved at 1 mg/mL in PBS containing 0.05% sodium azide and 5 U/ml hyaluronidase IV-S (H3884, Sigma). Following incubation at 37 °C overnight, the enzyme was heat-inactivated. The resulting digestion product (HA-MA) was dialyzed against deionized water for 48 h and lyophilized. The average molecular weight of the isolated HA-MA was determined to be ~400 kDa with a polydispersity of ~1.25 using gel permeation chromatography (Viscotek).

Synthesis of Acrylate-Derivatized Cell Adhesion Peptide—Cell adhesion peptide RGDS (American Peptide) was reacted with acryloyl-PEG-N-hydroxysuccinimide (ACRL-PEG-NHS; 3.4 kDa, Nektar) at a 1:1 molar ratio for 2 h in 50 mM sodium bicarbonate buffer, pH 8.5 [31]. The product (ACRL-PEG-RGDS) was purified by dialysis, lyophilized, and stored at –20 °C until use.

Cell Culture

Cryopreserved porcine vocal fold fibroblasts at passage 3 were obtained from Robert Langer, ScD (Massachusetts Institute of Technology, MIT) [32]. These cells had been isolated by primary explant from the mid-membranous lamina propria of 6–12 month old pigs, an accepted animal model for the human vocal fold lamina propria [3]. The discarded animal tissue was obtained via the MIT Division of Comparative Medicine and with the approval and according to the guidelines of the MIT animal care committee. The purity of the explant population was verified by positive staining for vimentin and negative staining for epithelial and endothelial cell-specific markers. The cryopreserved vocal fold fibroblasts were thawed and expanded at 37 °C/5% CO₂ in Dulbecco's Modified Eagle's Media (DMEM, Hyclone) supplemented with 10% fetal bovine serum (FBS), 2 ng/mL basic fibroblast growth factor (BD Biosciences), 100 U/mL penicillin and 100 mg/L streptomycin (Hyclone).

Cell Encapsulation and Hydrogel Maintenance

Hydrogels were fabricated by preparing precursor solutions containing 100 mg/mL 10 kDa PEGDA, 1.4 mg/ml of methacrylate-derivatized GAG (CSC-MA, HS-MA, or HA-MA), and 1.4 μmol/ml ACRL-PEG-RGDS in HEPES buffered saline (HBS; 10 mM HEPES, 150 mM NaCl, pH 7.4). The concentration of GAG was chosen to be consistent with GAG levels within the normal vocal fold lamina propria [22, 33]. In addition, PEGDA molecular weight and concentration were selected to yield hydrogels with average dynamic elastic moduli appropriate to vocal fold applications [9, 15].

A 300 mg/ml solution of UV photoinitiator 2,2-dimethoxy-2-phenyl-acetophenone in N-vinylpyrrolidone was added at 1 (v/v)% to each precursor solution. The resulting solutions were then poured into molds composed of two glass plates separated by 1.1 mm polycarbonate spacers and polymerized by 3 min exposure to longwave UV light (Spectroline, ~6 mW/cm², 365 nm). To generate cell-laden hydrogels, vocal fold fibroblasts at passage 5–6 were harvested and resuspended in the filter-sterilized precursor solutions at ~1×10⁶ cells/mL prior to transfer to the hydrogel mold. Polymerized hydrogel slabs were transferred to Omnitrays (Nunc) fitted with 4 sterile polycarbonate bars to simultaneously prevent gel flotation and prevent gel contact with the tray bottom. Hydrogels were immersed in DMEM supplemented with 10% FBS, 100 U/mL penicillin, and 100 mg/L streptomycin and maintained at 37 °C/5 % CO₂. Media was changed every two days.

Initial Hydrogel Characterization

Initial Water Uptake—To compare the extent of initial water uptake by the various hydrogel formulations following polymerization, the initial weight (W_i) of each gel ($n = 8$ per formulation) was determined immediately following polymerization, after which the gels were submerged in media. After 24 h, the swollen weight (W_s) of each gel was measured.

The equilibrium water uptake (S) was calculated as: $S = \left(\frac{W_s}{W_i} \right)$. This data was used to compare the post-swelling GAG levels across gels.

Average Mesh Size—PEGDA hydrogel mesh size cannot be readily visualized using standard techniques such as scanning electron microscopy. In the present study, hydrogel mesh size was therefore characterized via a series of dextran diffusion experiments based on an adaptation of the methodology of Watkins et al [34]. To avoid charge–charge interactions between the diffusing dextrans and the GAG chains in the hydrogel networks, fluorescently-labeled, charge-neutral dextrans were employed.

In brief, PEGDA-GAG hydrogels were prepared and allowed to swell overnight at 37 °C in PBS containing 0.05% azide (PBS-azide). One cm diameter discs were cored from each hydrogel formulation. Fluorescently-labeled, charge-neutral dextrans (40 kDa and 70 kDa, Invitrogen) were dissolved at 0.05 mg/mL in PBS-azide and added at 1 mL per hydrogel disc (8 discs per formulation per dextran molecular weight). Dextran solutions were allowed to diffuse into the hydrogels for 24 h at 37 °C. Each gel disc was gently blotted and transferred to 1 mL fresh PBS-azide. Dextran that had penetrated into the hydrogels was then permitted to diffuse out into the surrounding solution at 37 °C. After 24 h, the fluorescence of the PBS-azide solution surrounding each disc was measured at ex/em 530/590. Dextran standard curves were used to convert each fluorescence signal to a concentration. For each hydrogel formulation, the measured concentration readings for each dextran molecular weight were divided by gel weight and then plotted versus dextran hydrodynamic radius [35]. The area (A) under the resulting curve served as a quantitative indicator of the permissivity of each hydrogel (x) over the hydrodynamic radii range assayed. For the purposes of comparison, the calculated area values for CSC and HS gels

were normalized to that of the HA gels to yield a relative mesh size value: $\xi_x \sim \left(\frac{A_x}{A_{HA}} \right)$.

Hydrogel Mechanical Properties—Following initial swelling, 8 mm diameter discs ($n = 7$ –8 per formulation) were cored from each cell-laden hydrogel. The thickness of each disc was measured using a digital caliper. These thickness measures served both as gauge lengths for mechanical testing as well as indicators of hydrogel degradation and/or cell-mediated gel compaction with time. Each hydrogel disc was mounted onto the compression platens of a DMA 800 (TA Instruments) and surrounded at the edges by silicone oil to prevent gel

dehydration. Following application of a 0.01 N preload, each hydrogel disc was subjected to unconstrained compression at 1% cyclic strain at room temperature over a frequency range of 1–100 Hz (100 Hz being the system maximum). The higher frequency testing conditions were selected to approach the frequencies of cyclic lamina propria loading experienced during phonation, with the onset of adult phonation occurring at ~150 Hz in males. Sample elastic compressive modulus (E') and damping ratio ($\zeta = E''/E'$, where E'' = viscous modulus) were extracted from the resulting stress-strain data.

In comparing these compressive mechanical property data to literature values for the vocal fold lamina propria (which are generally obtained under shear), the fact that PEGDA hydrogels can be considered homogenous and isotropic was used [36–37]. Specifically, the elastic compressive modulus of homogenous, isotropic materials is related to the elastic shear modulus (G') by the following relationship: $G' = E'/(2+2\nu)$, where ν is the Poisson ratio. For polymeric hydrogels, $\nu \sim 0.5$, and thus, $G' = E'/3$ [37].

Endpoint Hydrogel Analyses

At day 18 of culture, gels were harvested for mechanical, biochemical, and histological analyses. Mechanical samples ($n = 3$ –5 per formulation) were tested as described above.

Biochemical Analyses—Samples harvested for biochemical analyses were transferred to screw-cap vials, weighed, flash-frozen in liquid nitrogen, and stored at -80°C until time of analysis. The weight of each 8 mm disc was used as a second indicator of gel degradation and/or compaction, since the reduction in gel volume associated with both of these processes would be reflected in a mass loss, assuming lack of commensurate neomatrix deposition [32]. Hydrogel samples were digested for 72 h at 37°C in 1 ml of 0.12 M NaOH per 0.2 g hydrogel wet weight [18, 38]. DNA, total collagen, and elastin were then analyzed per established protocols. For each assay, the standards used were subjected to the same association with PEGDA and GAG and to the same digestion conditions as the samples. Resulting collagen and elastin data were normalized to cell number as determined from the DNA measures.

DNA Analysis—Aliquots of the hydrolyzed samples ($n = 3$ –6 per formulation) were neutralized, and their DNA content determined using the Invitrogen PicoGreen assay [39]. DNA measures were translated to cell number using a conversion factor of 6.6 pg DNA per cell [40]. NaOH-exposed calf thymus DNA (Sigma) served as a standard.

Collagen Analysis—The amino acid hydroxyproline was quantified as an indirect measure of total collagen. In brief, aliquots ($n = 4$ –6 per formulation) collected for collagen quantitation were hydrolyzed for 18 h at 110°C in 6 M HCl. Each sample was then dried on a Labconco Centrivap to remove HCl, resuspended in deionized water, and passed through a charcoal-packed centrifugal microcolumn (Nunc) to remove caramelized sugars. The resultant samples were reacted with chloramine T and p-dimethylbenzaldehyde reagents [38]. Sample absorbance was read at 550 nm relative to that of L-4-hydroxyproline (Sigma). Total collagen content was estimated from measured grams of hydroxyproline by dividing by 0.13 [41].

Elastin Analysis—Elastin production was measured using indirect ELISA [42]. NaOH digested samples ($n = 3$ –4 per formulation) were neutralized and further digested with 0.25 M oxalic acid at 100°C overnight. Oxalic acid was removed from the samples and exchanged for PBS using Microcon YM-3 centrifugal filters (Millipore). One hundred μL of the resulting samples were applied to a high-binding multiwell plate for 3 h at room temperature. After blocking the plate with bovine serum albumin, adsorbed elastin

fragments were detected by applying elastin antibody (BA-4, Santa Cruz Biotechnology, [SCBT]) followed by donkey anti-mouse HRP secondary antibody (Jackson Immunochemicals) and 2,2'-azino-bis(3-ethylbenzthiazoline-6-sulphonic acid). Absorbance was read at 410 nm, with bovine aortic elastin (exposed to both NaOH and oxalic acid treatment, Sigma) serving as a standard.

Histological Analyses

Day 18 samples harvested for histological analyses were fixed in 10% formalin for 30 min, embedded in Tissue-Tek freezing media, and frozen at -80°C . Thirty-five μm sections were cut using a cryomicrotome. All immunostaining steps took place at room temperature unless otherwise noted.

Immunostaining—ECM deposition was analyzed for each formulation using standard immunohistochemical technique. Rehydrated sections were blocked with peroxidase for 10 min followed by 30 min exposure to Terminator (blocking agent, Biocare Medical). Primary antibodies for collagen I (Rockland Immunochemicals), collagen III (Rockland Immunochemicals), elastin (BA-4, SCBT), fibrillin-1 (C-19, SCBT), or fibronectin (C-20, SCBT) diluted in HBS were then applied for 1 h. To identify vocal fold fibroblasts displaying a myofibroblast-like phenotype, primary antibody for SM- α -actin (1A4, LabVision) was applied. Bound primary antibody was detected by using AP-conjugated secondary antibody (Jackson Immunochemicals) followed by application of the chromogen Ferangi Blue (Biocare Medical) or by using HRP-conjugated secondary antibody (Jackson Immunochemicals) followed by application of the chromogen AEC (LabVision). For cell signaling studies involving antibodies for pERK1/2 (Thr 202/Tyr 204, SCBT), pPLC- γ 1 (Tyr 1253, SCBT), NF κ B-p50 (C-19, SCBT), and NF κ B-p65 (C-20, SCBT), rehydrated sections were permeabilized (10 mM HEPES, pH 6.8, 100 mM NaCl, 3mM MgCl₂, 300 mM sucrose, 0.5% Triton X-100) for 30 min prior to Terminator application.

Semi-Quantitative Assessments—For intracellular markers (SM- α -actin, pERK1/2, pPLC- γ 1, NF κ B-p50, NF κ B-p65), cell counts were carried out to semi-quantitatively evaluate immunostaining results. These counting assessments were conducted according to established methods [43–45] on sections from at least 3 distinct discs (a total of ~1500 vocal fold fibroblasts). For each cell, i , in a given section, a staining intensity, d_i , was recorded on a scale of 0–3, 0 = “no staining” and 3 = “highest intensity among all formulations for that antibody” by a single observer blinded to outcome. Examples of individual cell intensity assignments are provided in Supplementary Figure 1. The cumulative staining intensity, d , for a given antibody in a particular section was calculated using the following equation: $d = (\sum d_i)/(\text{total cell number})$. In addition, since deposited ECM remained localized around the parent cells in each hydrogel formulation, as is characteristic for PEGDA gels [15], the relative levels of collagen I, collagen III, fibrillin-1, and fibronectin among hydrogel formulations were also evaluated by cell counts per the above procedure.

Counts for all stainings for which statistically-significant differences were observed among PEG-GAG formulations were repeated by two additional observers blinded to both sample identity and outcome. Each of these recounting assessments demonstrated at least 95% correlation with the original counts (Supplementary Table 2). The resulting average cumulative staining intensities (d_{avg}) for each antibody were regarded as vectors with CSC, HS, and HA components: $[d_{CSC} \ d_{HS} \ d_{HA}]$. For the purposes of comparison, these vectors

were normalized to have unit magnitude per standard methods:
$$\frac{[\ d_{CSC} \ d_{HS} \ d_{HA} \]}{\sqrt{d_{CSC}^2 + d_{HS}^2 + d_{HA}^2}}.$$

Comparison of Collagen I and Collagen III—Although the collagen I semi-quantitative assessments cannot give us the absolute amounts of collagen I in each formulation, they do bind the ratios between the collagen I levels among formulations. Similarly, the collagen III counting assessments bind the ratios between the collagen III levels among formulations. Given the predominance of collagen I and collagen III (> 90% of total collagen) in the vocal fold lamina propria [46], weighting factors for the collagen I and collagen III staining intensity vectors should exist for which the sum of the weighted vectors approximates the total collagen vector:

$$\left(\alpha_1 \begin{bmatrix} coll_{CSC} \\ coll_{HS} \\ coll_{HA} \end{bmatrix} + \alpha_3 \begin{bmatrix} colIII_{CSC} \\ colIII_{HS} \\ colIII_{HA} \end{bmatrix} \right) = \begin{bmatrix} totalcollagen_{CSC} \\ totalcollagen_{HS} \\ totalcollagen_{HA} \end{bmatrix}, \quad (1)$$

i.e. an α_1 and α_3 which minimize the error between the left and right hand sides of equation 1 can be determined. When the collagen I and collagen III vectors are first normalized to have unit magnitude prior to the determination of α_1 and α_3 , then the ratio of the resultant α_1 and α_3 can be interpreted as the relative contribution of the collagen I and collagen III

vectors to the overall total collagen vector. Thus, the $\frac{\alpha_1 \times coll_x}{\alpha_3 \times colIII_x}$ can be interpreted as the relative amount of collagen I to collagen III in formulation x . In the present study, α_1 and α_3 were determined to be 3.0×10^{-5} and 1.5×10^{-5} , respectively, using the Solver function of the Excel Analysis Toolbox, with the sum of the mean square error utilized as the error function to be minimized.

Statistical Analyses

Data are reported as mean $\pm$ standard deviation. Comparison of sample means was performed by ANOVA. Details regarding the number of ANOVAs performed as well as associated dependent variables are given in Supplementary Table 1. When readings for various measures could be attributed to the same sample group (e.g. 24 h elastic compressive modulus values at each sampled frequency), a single two-way ANOVA was performed in sample comparison. In the case of immunostaining, sections used for two different antibodies could not be considered as representing the same sample, even if arising from the same gel disc. Therefore, statistical comparisons for each antibody were performed separately using one-way ANOVA.

When the p-value associated with the independent-variable main effect fell below 0.05, pair-wise comparisons between specific formulations were then conducted using Tukey's post-hoc tests (two-tailed, SPSS software), $p < 0.05$. The significance value of each independent-variable main effect with $p < 0.05$ is given in Supplementary Table 2. The significance values associated with subsequent pair-wise comparisons are given in the text.

RESULTS

Hydrogel Material Properties

In order to attribute differences in vocal fold fibroblast behavior across hydrogel formulations specifically to initial differences in gel GAG composition, it was important that the remaining hydrogel material properties could be considered consistent across gels. The "bulk" material properties of each gel formulation were therefore characterized and compared. The initial water uptake, S , of each gel type following polymerization was similar (Table 1), indicating that the post-swelling concentration of GAG in each gel type was similar at (0.14/1.38) wt%, or ~ 0.1 wt% [30]. Similarly, comparison of the equilibrium

diffusion of charge-neutral dextrans indicated that bulk average mesh size of the swollen gel networks could be considered consistent across formulations (Table 1).

Mechanical property data for the PEG-GAG hydrogels over a frequency range of 1–40 Hz are summarized in Figure 1 and Table 2. Although mechanical measures were collected up to 100 Hz (approaching the onset of phonation at ~150 Hz), values above 40 Hz were neglected due to data quality limitations associated with testing device inertia. The average elastic compressive modulus values of both pure PEG and PEG-GAG gels were ~16 kPa at 1 Hz. As loading frequency increased, the average elastic compressive modulus values of both the pure PEG and PEG-GAG hydrogels increased to ~25 kPa (40 Hz, Figure 1). Given the homogenous and isotropic nature of PEG-based gels [36–37], these average elastic compressive modulus values can be assumed to correspond to an elastic shear modulus of ~8 kPa for both the pure PEG and PEG-GAG gels.

As the loading frequency increased, the differences in damping ratios between the GAG-containing and non-GAG containing gels increased, with the damping ratios of the PEG-CSC, PEG-HS, PEG-HA gels being significantly greater than that of the PEG gels at 40 Hz ($p = 0.0129$, $p = 0.046$; and $p = 0.013$, respectively). Specifically, the damping ratios of the PEG-GAG hydrogels were ~0.8 at 40 Hz, whereas pure PEG gels displayed a damping ratio of ~0.55 at 40 Hz. Elastic shear modulus and damping ratio ranges for the vocal fold lamina propria at similar strains and loading frequencies are 0.1–7 kPa and 0.6–2, respectively [47–48]. Thus, conjugation of GAGs into PEG networks allowed the hydrogels to more closely approach “average” lamina propria mechanical properties, at least in terms of observed damping ratios.

Mechanical property assessments at the study endpoint indicated that initial mechanical properties were maintained over 18 days of culture (Table 2). Similarly, the 24 h and 18 day thickness and mass data corresponded to negligible alterations in gel volume with time (Table 2). Thus, the bulk modulus, thickness, and gel mass assessments indicated that the PEG-GAG gels retained the slow degradation rate and resistance to cell-mediated contraction characteristic of pure PEG hydrogels [18].

Cell Density and ECM Deposition

Scarring is characterized by altered cell densities as well as altered deposition and contraction of ECM constituents. For example, increases in collagen I, collagen III, and fibronectin expression have been associated with vocal fold scar formation [3]. In addition, the density of the elastic microfibril, fibrillin-1, has been observed to be significantly reduced in normal and hypertrophic dermal scar relative to normal skin [49]. In the present study, endpoint cell densities within each hydrogel formulation were 65–70% of the initial seeding density, as is typical for cells photo-encapsulated within PEGDA hydrogels [50]. Similarly, per cell hydroxyproline assessments indicated that total collagen production was consistent across hydrogel formulations (Figure 2A).

Biochemical and histological assays also suggested that per cell elastin deposition could not be distinguished among gel types (Figure 2A, B). However, fibrillin-1 levels were significantly greater in HS ($p = 0.001$) and HA ($p = 0.034$) hydrogels than in CSC gels (Figure 2B), and fibronectin synthesis was higher in CSC than in HA gels ($p = 0.044$, Figure 3). Similarly, collagen I expression was significantly greater in CSC gels relative to HA ($p = 0.002$) and HS ($p = 0.021$) hydrogels, whereas collagen III was higher in HA gels than in CSC ($p < 0.001$) and HS ($p = 0.001$) hydrogels. Quantitative comparison of the present collagen I and III unit-magnitude intensity vectors (Figure 3) with the total collagen vector (Figure 2) indicated collagen I:collagen III ratios of approximately 12, 3, and 0.7 in the CSC, HS, and HA formulations, respectively. Representative images for collagen III

staining are given in Figure 4 and display the pericellular localization observed for each of the examined ECM proteins [15, 18]. Representative immunostaining images for the remaining ECM proteins analyzed are shown in Supplementary Figure 2.

Cell Phenotype and Signaling

Scarring and contracture are often promoted by resident fibroblasts taking on contractile, myofibroblast-like phenotypes [51]. Therefore, vocal fold fibroblast expression of myofibroblast-marker SM- α -actin was evaluated across hydrogel formulations (Figure 5). Levels of SM- α -actin were lower in HA hydrogels relative to CSC ($p < 0.001$) and HS ($p < 0.001$) gels. To gain insight into the intracellular signaling underlying observed vocal fold fibroblast phenotype and ECM production, signaling molecules activated by GAG interactions with cell surface receptors CD44 and RHAMM were investigated. As shown schematically in Figure 6, GAG interactions with these receptors activate a number of signaling molecules, including NF κ B, ERK1/2, and PLC γ 1, which in turn modulate cell gene expression [52–54]. In the present study, no significant differences in pPLC- γ 1 were observed among formulations (Figures 4 and 5). However, NF κ B-p50 levels were significantly higher in HS gels relative to HA ($p < 0.001$) and CSC ($p < 0.001$) gels (Figure 5), and NF κ B-p65 levels were greater in CSC hydrogels than in HA gels ($p = 0.041$). In addition, pERK1/2 levels were lower in HS gels relative to CSC hydrogels ($p = 0.036$). Representative immunostaining images for SM- α -actin, pERK1/2, NF κ B-p50, and NF κ B-p65 are shown in Supplementary Figure 2.

DISCUSSION

The present study was designed to examine the impact of CSC and HS relative to HA on vocal fold fibroblast ECM production and phenotype toward the improved design of implants for the treatment of superficial lamina propria (SLP) scar. For an implant to restore scarred vocal fold SLP and/or to inhibit further SLP fibrosis, it is critical that the scaffold not trigger fibrotic vocal fold fibroblast behaviors, such as the increased deposition of total collagen [25]. In the current work, vocal fold fibroblasts in hydrogels containing CSC and HS displayed similar levels of total collagen relative to vocal fold fibroblasts in gels containing HA. Given that HA has previously been associated with a reduction in fibrotic response for a range of tissue types [7, 11, 55], the similarity in total collagen production between CSC, HS, and HA gels may be advantageous in terms of SLP restoration. That said, marked differences in the relative production of collagens I and III were observed among hydrogel formulations, with CSC having the highest collagen I:collagen III ratio and HA having the lowest collagen I:collagen III ratio. As previously discussed, comparison of the present total collagen and collagen type data indicated that the ratio of collagen I:collagen III varied with formulation, from ~12 in the CSC gels to ~0.7 in the HA gels. Since the ratio of collagen I:collagen III in the vocal fold SLP is estimated to be less than 1.5 [46, 56], the current results suggest that the HA formulation resulted in collagen compositions most mimetic of normal SLP.

In normal human vocal fold lamina propria, fibrillin-1 tends to be enriched in the pliable SLP whereas elastin is essentially absent in this layer [57]. Fibrillin-1 has therefore been hypothesized to play a key role in SLP elasticity during phonation [24]. Although elastin deposition was similar across the PEG-GAG gel formulations, the HS hydrogels appeared to support the highest fibrillin-1:elastin ratio, a feature which may be desirable for lamina propria regeneration. However, in the absence of quantitative measures of fibrillin-1, definitive statements regarding which PEG-GAG formulation supports a more appropriate fibrillin-1:elastin ratio cannot be made. In contrast to elastin, fibronectin was moderately increased in CSC hydrogels relative to HA gels. Since fibronectin expression has been consistently observed to be heightened in scarred vocal fold lamina propria [3], the

increased fibronectin levels associated with CSC gels may be unfavorable for vocal fold scar treatment.

Expression of myofibroblast marker SM- α -actin decreased significantly from CSC to HS and from HS to HA hydrogels. Given the role of myofibroblasts in scar contraction and abnormal ECM synthesis [51], these results indicate that CSC and HS hydrogels may stimulate undesirable vocal fold fibroblast phenotypes. To gain insight into the internal cellular signaling underlying observed vocal fold fibroblast phenotype and ECM production, signaling molecules activated by GAG interactions with cell surface receptors CD44 and RHAMM were investigated. Although the signaling cascades initiated by GAGs and/or GAG-associated molecules (such as growth factors)[58] are complex, CD44- and RHAMM-activated intermediates were selected for investigation due to the fact that each GAG under investigation directly interacts with one or both of these receptors. Specifically, both HA and HS actively bind RHAMM [59], whereas both HA and CSC interact with CD44 [59–60] (although HS and CSC have over 10 fold lower affinities for RHAMM and CD44, respectively, than HA [60–61]).

Comparison of the SM- α -actin and signaling data suggests that the increased levels of SM- α -actin in CSC gels may be correlated with increased NF κ B-p65 and/or pERK1/2-related signaling. Previous research has shown that expression of SM- α -actin by dermal fibroblasts is regulated in part by activation of the ERK1/2 pathway [62]. Similarly, ERK1/2 activation has been associated with increased fibronectin expression by kidney myofibroblasts [63]. For HS hydrogels, the higher SM- α -actin levels relative to HA gels may be correlated with increased NF κ B-p50, a transcription factor which plays important roles in inflammatory and immune responses. This would be consistent with a previous study of myofibroblasts which found that suppressing NF κ B-p50 activation reduced expression of SM- α -actin as well as of collagen I [64].

Several limitations of the present study merit comment. The tight crosslink density and slow degradation rate of PEGDA gels induced associated cells to take on oval/stellate morphologies, despite the presence of cell adhesion ligand RGDS [15, 18]. These morphologies are non-native for vocal fold fibroblasts, which are normally elongated. However, a previous study has demonstrated that vocal fold fibroblasts encapsulated within PEGDA gels responded to matrix-mediated stimuli with similar trends (at least in terms of relative collagen, elastic fiber, and SM- α -actin expression) as vocal fold fibroblasts encapsulated in hydrogels permissive of more elongated cell morphologies [18]. An additional limitation of the present work results from the analysis of only a single concentration of each GAG type. Furthermore, the potential impact of the proteoglycan core protein or varying GAG chain length in modulating cellular interpretation of the applied GAG signals was not accounted for. Similarly, CSC and HS interact with a range of growth factors [65–69], and the potential effects of these factors on observed cell behavior were not assessed. In terms of analysis methods, many of the present conclusions are based on semi-quantitative immunostaining analyses and thus, results should be interpreted with caution. This is particularly true for the activated transcription factors examined, since the immunostaining assessments did not distinguish between nuclear-localized and cytoplasmic proteins.

That said, the cumulative ECM and phenotypic data indicate that the PEG-HA gels may be the most appropriate for vocal fold SLP regeneration of the formulations examined, at least for the GAG molecular weights investigated. Future studies will therefore focus on PEG-HA hydrogels and will explore a broader range of PEGDA molecular weights and concentrations in order to further improve the correspondence between the dynamic mechanical response of the PEG-HA gels and that of the vocal fold lamina propria.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

The dynamic compressive modulus and (B) damping ratio of the various PEG-GAG formulations compared to pure PEG gels. For each formulation, 7–8 cell-laden gels were tested. *, applies to each PEG-GAG gel and indicates a significance difference with the PEG hydrogel controls at 40 Hz, $p < 0.05$.

Figure 2.

Total collagen and elastic fiber production across hydrogel formulations. (A) Per cell hydroxyproline and ELISA-based assessments of total collagen and elastin, respectively. (B) Semi-quantitative, histological assessments of elastin and fibrillin-1. For the purposes of comparison, the elastin and fibrillin-1 cumulative staining intensities for each formulation were normalized to have unit magnitude. *, significantly different from CSC hydrogels, $p < 0.05$.

Figure 3.

Semi-quantitative histological assessments of collagen type and fibronectin production across hydrogel formulations. For the purposes of comparison, the cumulative staining intensities for each formulation for each antibody were normalized to have unit magnitude. *, significantly different from CSC hydrogels, $p < 0.05$; #, significantly different from HS hydrogels, $p < 0.05$.

Figure 4.

Representative images of CSC, HS, and HA hydrogel sections immunostained for collagen III and PLC-γ1. The scale bar in the lower right-hand image equals 40 μm and applies to all images. Representative images for remaining immunostainings are provided in Supplementary Figure 2.

Figure 5.

Semi-quantitative histological assessments of SM- α -actin, pERK1/2, NF κ B-p50, NF κ B-p65, and pPLC- γ 1 expression across hydrogel formulations. For the purposes of comparison, the cumulative staining intensities for each formulation for each antibody were normalized to have unit magnitude. *, significantly different from CSC hydrogels, $p < 0.05$; #, significantly different from HS hydrogels, $p < 0.05$.

Figure 6.
Schematic of RHAMM and CD44 signaling and associated signaling intermediates adapted from Turley et al [54].

Table 1

Hydrogel Water Uptake and Relative Mesh Size

	Water Uptake (S) [*]	Relative Mesh Size ^{*,†}
CSC	1.38 ± 0.02	1.01 ± 0.03
HS	1.38 ± 0.01	1.00 ± 0.01
HA	1.39 ± 0.01	1.00 ± 0.04

^{*} Eight specimens were tested for both water uptake and relative mesh size assessments.

[†] A relative mesh size of 1.00 corresponds to an average mesh size of ~18 nm [31].

Table 2

Hydrogel Modulus, Thickness, and Mass Assessments[†]

	Elastic Compressive Modulus (kPa) at 1 Hz		Thickness (mm)		Mass of 8 mm Discs (mg)	
	24 h	18 days	24 h	18 days	24 h	18 days
CSC	16.4 ± 2.7	19.1 ± 1.3	1.15 ± 0.04	1.16 ± 0.04	57.6 ± 0.9	59.5 ± 3.0
HS	17.4 ± 2.1	18.3 ± 2.5	1.15 ± 0.05	1.14 ± 0.07	57.6 ± 0.4	59.4 ± 3.4
HA	16.7 ± 2.0	17.5 ± 1.7	1.16 ± 0.03	1.17 ± 0.02	58.1 ± 0.2	58.7 ± 5.3

[†] Data were collected on the following numbers of cell-laden samples per formulation: Elastic compressive modulus, n = 7–8 (24 h) and n = 3–5 (18 days); thickness, n = 7–8 (24 h) and n = 3–5 (18 days); mass of discs, n = 8 (24 h) and n = 8–11 (18 days).