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Regeneration of Aged Rat Vocal Folds using Hepatocyte Growth Factor Therapy

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Abstract

Objectives/Hypothesis—We investigated acute changes in extracellular matrix gene expression and histologic changes in the deposition of collagen and hyaluronan (HA) from hepatocyte growth factor (HGF) treatment of the aged rat vocal fold. We hypothesized that: 1) HGF induces matrix metalloproteinase gene expression, which may contribute to the downregulation of collagen; and 2) HGF induces hyaluronan synthase (HAS) gene expression, which may play a role in the upregulation of ECM HA.

Study Design—prospective animal study.

Methods—Fifteen, 18-month old, Sprague-Dawley rats were involved in this study. For gene expression analyses, ten rats were divided into two groups and received serial injections of sham (saline) or HGF (2ng/μL) and sacrificed 2 weeks after the initial injection to investigate acute changes in extracellular matrix gene expression. A separate group of five animals received the above treatment and were sacrificed 4 weeks after the initial injection to investigate histologic changes in the deposition of collagen and HA.

Results—Real-time polymerase chain reaction revealed significantly upregulated MMP-2, -9, and HAS-3 messenger RNA (mRNA) expression and significantly downregulated procollagen type I mRNA expression in the HGF-treatment group, compared to the sham-treatment group. Histologic staining revealed significantly reduced collagen deposition and increased deposition of HA in the HGF-treated vocal fold, compared to the sham-treated vocal fold.

Conclusions—HGF induced the upregulation of MMP-2, -9, and HAS-3, and downregulated the expression of procollagen type I. Histologically, aged vocal folds treated with HGF revealed decreased collagen deposition, and increased deposition of HA, compared to sham-treated vocal folds.

Keywords

regenerative effect; hepatocyte growth factor; aged rat vocal fold; collagen; matrix metalloproteinase; hyaluronan

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INTRODUCTION

Age-related voice changes (presbyphonia) have been attributed to decreased efficiency of laryngeal motoneuron function, laryngeal muscle atrophy, laryngeal glandular degeneration, decreased pulmonary function, increased calcification of the laryngeal cartilages and alterations in the extracellular matrix (ECM) of the vocal fold lamina propria. Patients with presbyphonia often present with symptoms of reduced loudness, hoarseness, low pitch, tremor, breathiness, and vocal strain.¹ In early stages of presbyphonia, voice therapy is often used to reduce compensatory hyperfunction, improve vocal resonance, and to maximize respiratory support for phonation.² In more advanced cases, surgical augmentation and injection laryngoplasty are used to medialize the severely atrophic vocal fold.² Unfortunately, prevailing treatments for the aged vocal fold often serve as temporary space fillers, and do not fully address underlying changes to the ECM. Qualitative and quantitative changes occur within the aged vocal fold ECM, including a densely packed collagen network, an increase in the size of collagen fibrils, and decreased interstitial spaces for other extracellular matrices, such as hyaluronan (HA).^{3–5} Given our fundamental understanding of the ECM and the importance of tissue viscoelastic properties for phonation, treatments that target age-related changes of the ECM and regeneration of the vocal fold lamina propria may provide a useful adjunct in the surgeon's clinical armamentarium for the management of presbyphonia.

Disorders involving excess accumulation of the ECM provide reasonable targets for pharmacological interventions. Matrix metalloproteinases (MMPs) belong to a family of enzymes involved in turnover of the ECM.⁶ These MMPs and their physiological tissue inhibitors provide logical targets for peptide growth factors with anti-fibrotic properties. Our previous studies have revealed therapeutic effects of hepatocyte growth factor (HGF) on the stimulation of reparative processes after vocal fold injury.^{7–8} HGF is a potent inducer of ECM degrading enzymes, such as MMPs, which may provide a biochemical basis for these findings.⁹

The purpose of the current study was to investigate acute changes in ECM gene expression and histologic changes in the deposition of collagen and HA from HGF-treatment of the aged rat vocal fold. We hypothesized that 1) HGF induces matrix metalloproteinase gene expression, which may contribute to the downregulation of collagen in the aged vocal fold; and 2) HGF induces hyaluronan synthase (HAS) gene expression, which may play a role in the upregulation of ECM HA. Real-time reverse transcription polymerase chain reaction (RT-PCR) was used to quantify messenger RNA expression (mRNA) of MMP-2, -9, procollagen type I and HAS-2, -3 in HGF- and sham-treated vocal folds. Verhoeff's elastic and Alcian blue stains were used to investigate the deposition of collagen and HA from the HGF- and sham-treated vocal fold.

MATERIALS AND METHODS

Animals

Fifteen, 18-month old, male Sprague-Dawley rats were involved in this study. Rats of this age were chosen to represent elderly animals. Rats were obtained from Harlan Laboratories (Harlan Laboratories, Indianapolis, IN). In general, Sprague-Dawley rats live to be about 24 months old, and 50% of Sprague-Dawley rats will expire between 24–36 months of age. To assess for acute changes in ECM gene expression, 10 animals were equally and randomly assigned to receive bilateral vocal fold injections of HGF (100ng of HGF in 50 μ L, at a concentration of 2ng/ μ L) (HGF-treatment) or 50 μ L of phosphate buffered saline (PBS) (sham-treatment) and euthanized two weeks after initial treatment for analysis of gene expression and the testing of treatment group differences. To assess for histologic changes in

ECM deposition, the five remaining animals received a unilateral injection of HGF into one randomly selected vocal fold and sham-treatment into the contralateral vocal fold and were euthanized four weeks after initial treatment for histological comparisons between treatment sides.

Surgical Procedure

Animals received intramuscular injections of ketamine (60 mg/kg) and xylazine (6 mg/kg) at the time of anesthesia induction. Rats were positioned on a custom-made operating platform and the larynx was suspended using a custom-made spring-like device as reported previously.^{8,10} After adequate visualization of the vocal folds using a 1.9 mm, 30 degree telescope (KARL STORZ ENDOSCOPY-AMERICA, INC. Culver City, CA, USA), a 30 gauge needle was used to inject 50 μ L of HGF solution (100ng of HGF in 50 μ L, at a concentration of 2ng/ μ L) (HGF-treatment) or 50 μ L of phosphate buffered saline (PBS) (sham-treatment) into the lateral portion of the vocal fold. Because the lamina propria and thyroarytenoid muscle are not easily distinguished in the rat, we have found that the deep layer of the vocal fold is easier to define anatomically. Therefore, in order to increase the consistency of the injection procedure across rats, the needle tip is inserted into the deep portion of the vocal fold and diffusion of the solution is confirmed telescopically. Based on the displacement of the vocal fold medially during the injection, we believe that HGF solution diffuses into the lamina propria and do not believe there to be any untoward effects on muscle cells. To maximize solution bio-availability, a two week course of therapy was completed with three injections of sham or HGF administered during each treatment week. Rationale for the above concentration and treatment course were driven by therapeutic effects of HGF at the selected concentration and treatment course, as published previously.⁸ HGF was obtained commercially (PeproTech EC. Ltd, London). Whole larynges were harvested from animals following an overdose intracardiac injection of pentobarbital sodium (150 mg/kg).

Real-time PCR

Vocal fold specimens were dissected away from the larynx. A Mixer Mill MM 301 (Retsch Inc. Pittsburgh, PA, USA) was used to homogenize vocal fold specimens. Total RNA was isolated using an RNeasy Mini Kit (Qiagen, Valencia, CA, USA) and treated with ribonuclease-free deoxyribonuclease I (QIAGEN, Valencia, CA, USA) to minimize contamination from genomic DNA. The quantity of total RNA was determined using the A260/A280 ratio and electrophoresis was used to evaluate the quality based on the appearance of the 18S and 28S ribosomal RNA bands. Reverse transcription was performed using a High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA, USA) using the manufacturers recommended reaction protocol. Reactions were performed with a Biometra TGradient Thermocycler (LABREPCO, Horsham, PA, USA) using the following parameters: 25°C for 10 min, 37°C for 120 min, 85°C for 5 sec, and 4°C for 5 min.

Rat-specific primers for MMP-2, -9, procollagen type I, HAS-2, -3, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were synthesized by Integrated DNA Technologies (Coralville, IA, USA) based on previously published sequences.^{8,11-14} Specific primer sequences are displayed in Table 1. All primers generated a single PCR band of the expected size. DNA sequencing was used to verify the PCR products. Real-time PCR was performed using the iQTMSyBR Green Supermix Kit (BioRad, CA, USA) in a 25 μ L volume reaction mixture composed of 500 nM primer1, 500 nM primer 2, 12.5 μ L iQ SyBR Green Supermix, 1 μ L of template complementary DNA, and 9 μ L of ribonuclease-free water. The following protocol was used for real-time PCR: 1 cycle at 95°C for 3 min, followed by 45 cycles at 95°C for 20 seconds, 58.7°C for 20 seconds, 72°C for 40 seconds, and 1 cycle at

95°C for 2 min, 55°C for 2 min, and then 1 cycle at 55°C to 95°C in 0.2°C increments to make a melting curve. An iCycler iQ™ Optical System (Software version 2.0; Bio-Rad, Hercules, CA, USA) was used to detect the PCR products. PCR products were separated by electrophoresis in 1% agarose gels containing 0.5µg/mL ethidium bromide for verification of PCR products according to fragment size.

Standard curves were used to determine the relative ratio of gene expression. Target gene ratios from sham- and HGF-treated vocal folds were normalized using expression ratios of the internal control gene (GAPDH). Bilateral vocal fold specimens were averaged for each animal before doing the group analysis with five animals per group. Non-parametric independent two-group comparisons were performed with the Mann-Whitney *U* test using log-transformed expression ratios. To assess for differences between sham- and HGF-treatment groups, separate Mann-Whitney *U* tests were performed for each gene with the appropriate alpha correction of .01 to control for type I error. All analyses were performed with the use of 2-tailed p-values. Data were analyzed using SPSS 15.0 for Windows (SPSS Inc., Chicago, IL, USA).

Histological Study

Whole larynges were soaked in embedding medium (OCT compound, Tissue-Tek, Torrance, CA, USA), frozen quickly using a combination of acetone and dry ice, and stored in a deep freezer (−80°C) for later analysis. In preparation for histology, frozen sections were made using ten-micron thick serial sections prepared in the coronal plane. Sections were prepared systematically from the posterior to the anterior portion of the vocal fold. Collagen was identified using Verhoeff's elastic stain. The hyaluronidase digestion technique was used for identification of HA. For the hyaluronidase digestion procedure, 50 mg of bovine testes hyaluronidase (Sigma, St Louis, MO, USA) was diluted in 100ml PBS solution, and slides were incubated in this solution for 1 hour at 37°C. Slides were then stained with Alcian blue (pH 2.5). HA was detected by comparing the slides receiving enzymatic digestion to those slides without enzymatic treatment. Images were captured with a Leica CTR 6500 microscope (Leica Microsystems Inc., Bannockburn, IL, USA). Stained regions in the lamina propria were measured using Adobe Photoshop Image Analysis Software (Adobe Systems Inc., San Jose, CA, USA), and image analysis was performed to compare differences in the mean staining intensity between HGF- and sham-treated vocal folds. The mean staining intensity was determined by measuring the total stained area using Image J (National Institutes of Health, Bethesda, MD, USA), and normalizing the total stained area with the total pixel area in the whole lamina propria. The image analysis procedure involves having the rater select an area of interest. The rater then uses the computer to count pixels within the stained region of the lamina propria. The computer provides information on the total number of pixels in the selected stained area, which the rater can then normalize with the pixel count throughout the entire vocal fold (stained and unstained). Intra-judge and inter-judge reliability was separately assessed using Spearman's correlation coefficients. Parametric dependent two-group comparisons were performed using Paired t-tests to investigate differences in the mean staining intensity between HGF- and sham-treated vocal folds. Alpha was adjusted to .025 to control for type I error. All analyses were performed with the use of 2-tailed p-values. Data were analyzed using SPSS 15.0 for Windows (SPSS Inc., Chicago, IL, USA).

RESULTS

PCR data

Results from Mann-Whitney *U* revealed a significant difference for MMP-2 ($p = .009$) (Figure 1A), MMP-9 ($p = .009$) (Figure 1B), procollagen type I ($p = .009$) (Figure 1C), and

HAS-3 ($p = .009$) (Figure 1E) expression between sham- and HGF-treatment groups. Specifically, MMP-2, -9, and HAS-3 expression were significantly increased in the HGF-treatment group, compared to the sham-treatment group ($p = .009$) (Figure 1A, 1B, and 1E) and procollagen type I was significantly decreased in the HGF-treatment group, compared to the sham-treatment group ($p = .009$) (Figure 1C). Mann-Whitney U revealed a non-significant increase in HAS-2 expression ($p = .016$) (Figure 1D) between sham- and HGF-treatment groups.

Histological data

Collagen was present throughout all layers of the sham- and HGF-treated vocal fold, with dense staining in the deep layer of the lamina propria in the sham-treated vocal fold (Figure 2A and 2C), and decreased density of staining in the HGF-treated vocal fold (Figure 2B and 2D). HA was present throughout all layers of the sham-treated (Figure 3A and 3C) and HGF-treated vocal fold (Figure 3B and 3D), with increased density of staining in the HGF-treated vocal fold, compared to the sham-treated vocal fold.

Results of the paired-t-test for collagen revealed that the staining density of collagen was significantly lower in the HGF-treated vocal fold, compared to the sham-treated vocal fold ($p < .0001$) (Figure 4). Results of the paired-t-test for HA revealed that the staining density of HA was significantly higher in the HGF-treated vocal fold, compared to the sham-treated vocal fold ($p = .014$) (Figure 4). Inter-rater reliability was performed on 10% of the slides, selected at random. Initial measures were correlated with repeated measures, revealing a Spearman Correlation Coefficient, $r = 1.00$. For intra-rater reliability, 10% of these slides were selected at random and remeasured by the same examiner. Initial measures were correlated with repeated measures, revealing a Spearman Correlation Coefficient, $r = 1.00$.

DISCUSSION

The vocal folds are composed of a unique amalgamation of fibrous proteins, interstitial proteins, and ground substance. The distribution and deposition of these extracellular matrices is important and contributes to the tissue's viscoelastic properties. These extracellular matrices are involved in the regulation of thickness of the lamina propria, fluid content, and they provide the lamina propria with structural support.^{3–5} Age-related morphological changes of the vocal fold are thought to result in presbyphonia. Both qualitative and quantitative changes take place within the aged vocal fold; notably an increase in the collagen network, and decreased interstitial spaces for extracellular matrices, such as hyaluronan.^{3–5} These age-related changes may lead to less than optimal viscoelastic tissue properties for phonation. Because of the important functions of collagen and HA and their relative contributions to tissue viscoelastic properties,^{15–17} therapeutic agents that target the underlying age-related changes of the ECM may offer a useful approach for restoration of the connective tissue changes associated with aging of the vocal fold lamina propria.

The purpose of the current study was to investigate the effects of HGF on acute changes in MMPs, procollagen, and HAS gene expression and histologic alterations in the deposition of collagen and HA in the aged rat vocal fold. In a reparative setting that closely resembles the age-related structural changes of the vocal fold ECM, our previous studies have revealed reduced tissue collagen,⁷ downregulated procollagen type I and TGF- β 1, and increased HAS-2 gene expression from injured vocal folds treated with HGF.⁸ These therapeutic effects in the context of acute and chronic vocal fold injury and the histologic similarities between chronic injury and age-related changes of the vocal fold ECM, have led us to pursue growth factor therapy for targeting the underlying age-related changes of the vocal fold lamina propria. We hypothesized that 1) HGF induces matrix metalloproteinase gene

expression, which may contribute to the downregulation of collagen in the aged vocal fold; and 2) HGF induces HAS gene expression, which may play a role in the upregulation of ECM HA.

Results revealed that MMP-2 and -9 gene expression were significantly increased and procollagen type I expression was significantly decreased in aged vocal folds receiving HGF-treatment, compared to sham-treated vocal folds. Histologically, the density of collagen staining was significantly lower in aged vocal folds receiving HGF-treatment, compared to sham-treated vocal folds. We suspect that the anti-fibrotic properties of HGF are mediated in part by MMP proteolysis, as reported in the lung.¹⁸ Results of the current study showed evidence of MMP gene upregulation following HGF treatment of the aged rat vocal fold. The MMP family of zinc-containing endopeptidases play an important role during tissue remodeling, creating space for cells to migrate during proteolysis, producing specific substrate-cleavage fragments, regulating tissue architecture, and activating/deactivating the activity of signaling molecules.⁶ MMP-2 (gelatinase A) has type IV collagenolytic activity and MMP-9 (gelatinase B) has type V collagenolytic activity.^{19–20} These MMPs are considered to be two key enzymes in wound healing and tissue remodeling. Importantly, MMP-2 and -9 are involved in the turnover of collagen fibrils in the extracellular space. In the aged vocal fold, it is believed that the overaccumulation of collagen may be responsible for changes to the tissue's viscoelastic properties.^{3–5} These changes may lead to less than optimal tissue properties for phonation, and result in dysphonia. Therefore, techniques aimed at reducing the amount of collagen in the aged vocal fold may lead to novel approaches in the restoration of age-related vocal fold changes. In the current study, the finding of increased MMP gene expression in the context of decreased density of collagen staining appears to provide support for the role of HGF in stimulating MMP proteolysis. Further studies are needed to determine whether increased turnover of collagen results in improved viscoelastic tissue properties.

Results from the testing of the second hypothesis revealed that HAS-3 gene expression was significantly higher in aged vocal folds receiving HGF-treatment, compared to sham-treated vocal folds. HAS-2 was non-significantly increased in HGF-treated vocal folds, compared to sham-treated vocal folds. Histologically, the density of HA staining was significantly upregulated in aged vocal folds receiving HGF-treatment, compared to sham-treated vocal folds. Previously, Hirano et al. reported increased cellular production of HA from aged vocal fold fibroblasts cultured with HGF.²¹ Results of the current study provide support from an *in-vivo* animal model for the effects of HGF-treatment on ECM HA production, which may be mediated in part by the upregulation of genes coding hyaluronan synthases. In the vocal fold, HA resides among the interstitial spaces sandwiched between collagens and elastin. HA contributes to tissue viscosity and provides slippage between molecules. Our previous studies have revealed functional consequences of reduced HA levels in the vocal fold after injury. Specifically, decreased HA is associated with increased phonation threshold pressure and decreased vocal economy.¹⁷ In the current study, the finding of increased HAS-3 gene expression in the context of increased density of HA staining, provides support for the role of HGF in stimulating HA production. Because HA is a chief contributor to tissue viscosity, restoration of age-related HA alterations may be useful in improving the viscoelastic properties of the aged vocal fold. It may also be possible that HGF has an effect on hyaluronidase gene expression (HA degrading enzymes). It would be interesting in future studies to investigate whether HGF downregulates tissue hyaluronidases, which would have the effect of slowing down turnover of HA in the aged vocal fold. However, further studies are needed with longer follow-up time points to investigate chronic histologic changes in the deposition of collagen and HA and the longevity of HGF-treatment effects.

CONCLUSION

The regenerative effects of HGF therapy on treatment of the aged rat vocal fold were investigated in the current study. HGF-treatment induced the upregulation of MMP-2, -9, and HAS-3, and downregulated the expression of procollagen type I. Histologically, aged vocal folds treated with HGF revealed decreased collagen deposition, and increased deposition of HA, compared to sham-treated vocal folds.

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This study was performed in accordance with the PHS Policy on Humane Care and Use of Laboratory Animals, the NIH *Guide for the Care and Use of Laboratory Animals*, and the Animal Welfare Act (7 U.S.C. et seq.); the animal use protocol was approved by the Institutional Animal Care and Use Committee (IACUC) of Vanderbilt University Medical Center.

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Fig 1.

Log-transformed mRNA expression ratios between the HGF-treatment group and the sham-treatment group for (A) matrix metalloproteinase (MMP)-2, (B) MMP-9, (C) procollagen type I, (D) hyaluronan synthase (HAS)-2, and (E) HAS-3, two-weeks after the initial treatment. The error bars represent 95.0% confidence intervals of the mean.

Figure 2

Fig 2.
Coronal sections of the sham (A,C) and HGF treated vocal fold (B,D) stained with Verhoeff's elastic stain for collagen, revealing decreased density of collagen staining in the lamina propria of the HGF-treated vocal fold, compared to the sham-treated vocal fold. Bars represent 100µm.

Fig 3.
Coronal sections of the sham (A,C) and HGF treated vocal fold (B,D) stained with Alcian blue stain for HA, revealing increased density of HA staining in the lamina propria of the HGF-treated vocal fold, compared to the sham-treated vocal fold. Bars represent 100 μ m.

Fig 4. Percent density of collagen and HA staining between the sham and HGF-treated vocal fold. Data are shown as mean $\pm$ SD (n=5). Results revealed significantly lower density of collagen staining and increased density of HA staining in the HGF-treated vocal fold, compared to the sham-treated vocal fold.

Table I**Primer Sequences.**

MMP-2	Forward	5'-GTC ACT CCG CTG CGC TTT TCT CG-3'
	Reverse	5'-GAC ACA TGG GGC ACC TTC TGA-3'
MMP-9	Forward	5'-CGG AGC ACG GGG ACG GGT ATC-3'
	Reverse	5'-AAG ACG AAG GGG AAG ACG CAC ATC-3'
Procollagen type I	Forward	5'- AAG GGT GAG ACA GGC GAA CAA -3'
	Reverse	5'- TTG CCA GGA GAA CCA GCA GAG -3'
HAS-2	Forward	5'-CCA ATG CAG TTT CGG TGA TG-3'
	Reverse	5'-ACT TGG ACC GAG CCG TGT AT-3'
HAS-3	Forward	5'-CCT CAT CGC CAC AGT CAT ACA A-3'
	Reverse	5'-CCA CCA GCT GCA CCG TTA GT-3'
GAPDH	Forward	5'-GAG TCA ACG ATT TGG TCG T-3'
	Reverse	5'-GAC AAG CTT CCC GTT CTC AG-3'

MMP – Matrix metalloproteinase; HAS – Hyaluronan synthase; GAPDH – Glyceraldehyde-3- phosphate dehydrogenase.