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Regenerative Effects of basic Fibroblast Growth Factor on Extracellular Matrix Production in Aged Rat Vocal Folds

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Abstract

Objectives—To investigate acute changes in extracellular matrix (ECM) gene expression and histologic changes in the deposition of collagen and hyaluronan (HA) from basic fibroblast growth factor (bFGF) treatment of the aged rat vocal fold.

Methods—For the polymerase chain reaction (PCR) experiments, ten 18 month old, Sprague-Dawley rats were divided into two groups and received serial injections of sham (saline) or bFGF (2ng/μL) and euthanized 2 weeks after the initial injection to investigate acute changes in ECM gene expression. A separate group of five animals received treatment and were sacrificed 4 weeks after the initial injection to investigate histologic changes in the deposition of collagen and HA.

Results—Real-time PCR revealed significantly upregulated hyaluronan synthase (HAS) -2, -3, matrix metalloproteinase (MMP) -2, and procollagen type I gene expression in the bFGF-treatment group, compared to the sham-treatment group. Histologic staining revealed significantly increased deposition of HA in the bFGF-treated vocal fold, compared to the sham-treated vocal fold. No differences in ECM collagen were observed between treatment sides.

Conclusions—Basic fibroblast growth factor induced the upregulation of HAS -2, -3, MMP -2, and procollagen type I. Histologically, aged vocal folds treated with bFGF revealed increased deposition of HA, compared to sham treated vocal folds.

Keywords

regenerative effect; aged vocal fold; basic fibroblast growth factor; matrix metalloproteinase; procollagen type I; hyaluronan synthase

Introduction

Age-related alterations in the extracellular matrix (ECM) of the vocal fold may result in structural changes to the lamina propria and play a role in presbyphonia.¹ Histologically, the aged vocal fold lamina propria has been characterized by excessive collagen deposition, thick bundle collagen formation, and reduced hyaluronan (HA).²⁻⁴ Although various approaches

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have been used to manage the symptoms associated with presbyphonia, treatments that address the underlying changes associated with aging of the vocal fold are in short supply. Fortunately, recent advances in our understanding of age-related vocal fold changes may lead to breakthroughs in the treatment of presbyphonia.

In the first human case treated with basic fibroblast growth factor (bFGF), Hirano et al revealed improved mucosal wave and glottic closure in a 63-year-old male with atrophic vocal folds.⁵ Basic fibroblast growth factor is a strong promoter of fibroblast proliferation and stimulates the production of HA from vocal fold fibroblasts in cell culture.⁶ Intracordal injections of bFGF have been shown to increase the density of ECM HA.⁷ However, the cellular mechanisms regulating the therapeutic effects of bFGF on the aged vocal fold remain unknown. Thus, 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 bFGF-treatment of the aged rat vocal fold. We hypothesized that: 1) bFGF induces hyaluronan synthase (HAS) gene expression, which may play a role in the upregulation of ECM HA; and 2) bFGF stimulates matrix metalloproteinase gene expression, which may contribute to increased turnover of collagen in the aged vocal fold. Real-time reverse transcription polymerase chain reaction (RT-PCR) was used to quantify messenger RNA expression (mRNA) of hyaluronan synthase (HAS)-2, -3, matrix metalloproteinase (MMP)-2, -9, and procollagen type I from bFGF- and sham-treated vocal folds. Histologic staining was used to investigate differences in the deposition of collagen and HA from bFGF- and sham-treated vocal folds.

Materials and Methods

Animals

Fifteen, 18 month old, male Sprague-Dawley rats were used in this study. Rats were obtained from Harlan Laboratories (Harlan Laboratories, Indianapolis, IN). According to the vendor, Sprague-Dawley rats live to be about 24 months old, and 50% of Sprague-Dawley rats will expire between 24-36 months of age. Eighteen month old rats were selected in the current study to represent elderly animals. To assess for acute changes in ECM gene expression, 10 animals were equally and randomly assigned to receive bilateral vocal fold injections of bFGF (100ng of bFGF in 50 μ L, at a concentration of 2ng/ μ L) (bFGF-treatment) or 50 μ L of phosphate buffered saline (PBS) (sham-treatment). These animals were euthanized two weeks after the initial injection 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 bFGF into one randomly selected vocal fold and sham-treatment into the contralateral vocal fold. These animals were euthanized four weeks after the initial injection for histological comparisons between treatment sides.

Surgical Procedure

Animals received intramuscular injections of ketamine (60 mg/kg) and xylazine (6 mg/kg) for induction of anesthesia. A custom-made operating platform was used to position rats in a near-vertical position.⁸ The larynx was suspended using a custom-made spring like device and the vocal folds were visualized using a 1.9 mm, 30 degree telescope (KARL STORZ ENDOSCOPY-AMERICA, INC. Culver City, CA, USA).⁹ After adequate visualization of the vocal folds, a 30 gauge needle was used to inject 50 μ L of bFGF solution (100ng of HGF in 50 μ L, at a concentration of 2ng/ μ L) (bFGF-treatment) or 50 μ L of phosphate buffered saline (PBS) (sham-treatment) into the lateral portion of the vocal fold. To maximize solution bio-availability, a two week course of treatment was completed with two injections of sham or bFGF administered during each treatment week. Basic fibroblast growth factor was obtained commercially (PeproTech EC. Ltd, London). Whole larynges were harvested from animals following an overdose intracardiac injection of pentobarbital sodium (150 mg/kg).

PCR Study

Vocal fold specimens were dissected away from the larynx under a light microscope. A Mixer Mill MM 301 (Retsch Inc. Pittsburgh, PA, USA) was used to homogenize vocal fold specimens. An RNeasy Mini Kit (Qiagen, Valencia, CA, USA) was used to isolate total RNA and treated with ribonuclease-free deoxyribonuclease I (QIAGEN, Valencia, CA, USA) to minimize contamination from genomic DNA. The A260/A280 ratio was used to determine the quantity of total RNA. Electrophoresis was used to evaluate the quality of RNA based on the appearance of the 18S and 28S ribosomal RNA bands. A High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA, USA) was used to perform reverse transcription using the manufacturers recommended reaction protocol. A Biometra TGradient Thermocycler (LABREPCO, Horsham, PA, USA) was used to perform reactions at: 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 HAS-2, -3, MMP-2, -9, procollagen type I, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were synthesized by Integrated DNA Technologies (Coralville, IA, USA) and used for PCR.⁸ Table 1 displays primer sequences. All primers generated a single PCR band of the expected size and PCR products were verified by DNA sequencing. The iQTMSyBR Green Supermix Kit (BioRad, CA, USA) was used to perform real-time PCR in a 25 µL volume reaction mixture composed of 500 nM primer1, 500 nM primer 2, 12.5 µL iQ SyBR Green Supermix, 1 µL of template complementary DNA, and 9 µL 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. PCR products were detected using an iCycler iQTM Optical System (Software version 2.0; Bio-Rad, Hercules, CA, USA). Electrophoresis was used to separate PCR products in 1 % agarose gels containing 0.5µg/mL ethidium bromide for verification of PCR products.

Standard curves were used to determine the relative ratio of gene expression. Target gene ratios from sham- and bFGF- treated vocal folds were normalized by the ratios of the internal control gene (GAPDH). Non-parametric independent two-group comparisons were performed using the Mann-Whitney *U* test using log-transformed expression ratios. To assess for differences between sham- and bFGF-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. 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 with a combination of acetone and dry ice, and stored in a deep freezer (-80°C). Frozen sections were made using ten-micron thick serial sections prepared in the coronal plane. All sections were made in a similar fashion, starting from the posterior to the anterior portions of the vocal fold. Collagen was identified using Verhoeff's elastic stain. The hyaluronidase digestion technique was used to detect HA. For the hyaluronidase digestion procedure, 50 mg of bovine testes hyaluronidase (Sigma, St Louis, MO, USA) was diluted in 100ml PBS solution, and each section was incubated in this solution for 1 hour at 37°C. The sections were then stained with Alcian blue (pH 2.5). HA was detected by comparing slides with and without enzymatic treatment. A Leica CTR 6500 microscope (Leica Microsystems Inc., Bannockburn, IL, USA) was used to digitize the slides. The stained regions in the lamina propria were measured using Adobe Photoshop Image Analysis Software (Adobe Systems Inc., San Jose, CA, USA), and analysis was performed to compare differences in the mean staining intensity between bFGF- 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 entire lamina propria. The total stained area included the entire lamina propria above the muscle layer. Sub-regions of the lamina propria were not investigated. Measurements were made by two investigators in a blinded fashion regarding experimental condition. 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 treatment sides. Alpha was adjusted to .025 to control for type I error. All analyses were performed with the use of 2-tailed p-values, and the data were analyzed using SPSS 15.0 for Windows (SPSS Inc., Chicago, IL, USA).

Results

PCR data

Results from Mann-Whitney *U* revealed significant between-group differences for HAS-2 ($p = .009$) (Figure 1A), HAS-3 ($p = .009$) (Figure 1B), MMP-2 ($p = .009$) (Figure 1C), and procollagen type I ($p = .009$) (Figure 1E). Results revealed that gene expression levels of HAS-2, -3, MMP-2 and procollagen type I were significantly upregulated in the bFGF-treatment group, compared to the sham-treatment group ($p = .009$) (Figure 1A, 1B, 1C and 1E). No measurable differences in MMP-9 gene expression were found between treatment groups. (Figure 1D).

Histological data

HA was present throughout all layers of the sham- and bFGF- treated vocal fold, with denser staining in the deep layer of the lamina propria in the bFGF-treated vocal fold, compared to the sham-treated vocal fold (Figure 2A, 2B, 2C, and 2D). In contrast, collagen was present throughout all layers of the sham- and bFGF- treated vocal fold, and showed no differences between treatment sides (Figure 3A and 3B).

Results of the paired t-test for HA revealed that the density of HA was significantly higher in the bFGF-treated vocal fold, compared to the sham-treated vocal fold ($p < .0001$) (Figure 4). Results of the paired t-test for collagen revealed no significant differences in the density of collagen staining between treatment sides ($p = .036$) (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

Age related changes in the deposition of collagen and HA in the vocal fold may result in less than optimal tissue properties for phonation. These age related changes have been attributed to a slowdown in the turnover of the ECM.¹⁰ Treatments that stimulate turnover of the ECM may provide a useful approach in the management of these age related changes. In recent years, therapeutic growth factors capable of stimulating the production of key ECM components and matrix degrading enzymes have been identified as promising treatments for the management of vocal fold ECM disorders.^{5-7,9} However, the molecular mechanisms underlying the effects of these growth factors have not been fully elucidated.

In the current study, we investigated acute changes in ECM gene expression and histologic changes in the deposition of collagen and HA from bFGF treatment of the aged rat vocal fold.

We hypothesized that: 1) bFGF induces HAS gene expression, which may play a role in the upregulation of ECM HA; and 2) bFGF stimulates matrix metalloproteinase gene expression, which may contribute to increased turnover of collagen in the aged vocal fold. Results revealed that the expression of HAS-2, and HAS-3 were significantly upregulated in aged vocal folds receiving bFGF-treatment, compared to sham-treated vocal folds. Histologically, the density of ECM HA was significantly increased in bFGF-treated vocal folds. In a preliminary report of bFGF treatment of the aged rat vocal fold, Hirano et al. reported increased density of HA staining in aged vocal folds treated with bFGF, compared to sham treatment.⁷ Although gene expression changes were not investigated in the earlier investigation, it was hypothesized that these effects might be mediated by increased cellular production of HA, based on observations from fibroblasts cultured in the presence of bFGF.⁶ Results from the current study provide *in-vivo* evidence of bFGF treatment effects on acute changes in HAS gene expression and ECM HA deposition. Results suggest that the effects of increased ECM HA may be mediated in part via a mechanism involving the upregulation of HAS gene expression.

Results also revealed that the expression of MMP-2 and procollagen type I expression were significantly upregulated in aged vocal folds receiving bFGF-treatment, compared to sham-treated vocal folds. Albeit, there were no significant changes in ECM levels of collagen between bFGF- and sham-treated vocal folds. Given the effects of MMPs on turnover of extracellular collagen, we hypothesized that bFGF upregulation of MMP gene expression would contribute to decreased levels of collagen in the aged vocal fold.¹¹⁻¹³ We suspect that the concurrent upregulation of procollagen type I expression (suggestive of active collagen synthesis) may have negated the effects of proteolytic digestion, resulting in the absence of a net change in ECM collagen. Further studies will be necessary to explore this hypothesis, as ECM production is a tightly regulated process involving not only a balance between ECM synthesis and degradation, but the suppression of matrix degradation by tissue inhibitors of metalloproteinases.

Because the process of protein formation in the vocal fold from transcription is unknown, in the present study we selected a two week time period to determine whether the change in gene expression could be measured histologically. However, the process of transcription, translation, and functional protein formation may take place in only a few days, or it may require a longer time period than the one selected for the present investigation. It would be interesting in future studies to investigate shorter follow up time periods. It may also be interesting to investigate longer follow up periods to determine whether the increase in procollagen gene expression eventually results in a histological change in ECM collagen with time.

Conclusion

Basic fibroblast growth factor treatment induced the upregulation of HAS-2, -3, MMP-2, and procollagen type I gene expression. Histologically, aged vocal folds treated with bFGF revealed increased deposition of HA, compared to sham-treated vocal folds. Despite alterations in MMP and procollagen gene expression, no differences in the levels of ECM collagen were found between the bFGF-treated and sham-treated vocal fold.

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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 bFGF-treatment and sham-treatment groups for (A) hyaluronan synthase (HAS)-2, (B) HAS-3, (C) matrix metalloproteinase (MMP)-2, (D) MMP-9, and (E) procollagen type I. Error bars represent 95.0% confidence intervals of the mean.

Fig 2.

Coronal sections of the sham (A,C) and bFGF-treated vocal fold (B,D) stained with Alcian blue for hyaluronan (HA). Results revealed increased density of HA in the lamina propria of the bFGF-treated vocal fold, compared to the sham-treated vocal fold. Bars represent 100μm.

Fig 3.
Coronal sections of the sham and bFGF-treated vocal fold stained with Verhoeff's elastic stain for collagen. Results revealed no differences in the density of collagen staining between treatment sides. Bars represent 100µm.

Fig 4. Percent density of HA and collagen staining between the sham- and bFGF-treated vocal fold. Results revealed significantly increased density of HA staining between treatment sides ($p < .0001$). Data are shown as mean $\pm$ SD ($n=5$).

Table 1**Primer Sequences**

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'
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'
GAPDH	Forward	5'-GAG TCA ACG ATT TGG TCG T-3'
	Reverse	5'-GAC AAG CTT CCC GTT CTC AG-3'

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