Homeostasis of Hyaluronic Acid in Normal and Scarred Vocal Folds

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Summary: Objectives/Hypothesis. Vocal fold scarring is one of the most challenging laryngeal disorders to treat. Hyaluronic acid (HA) is the main component of lamina propria, and it plays an important role in proper vocal fold vibration and is also thought to be important in fetal wound healing without scarring. Although several animal models of vocal fold scarring have been reported, little is known about the way in which HA is maintained in vocal folds. The purpose of this study was to clarify the homeostasis of HA by examining the expression of hyaluronan synthase (Has) and hyaluronidase (Hyal), which produce and digest HA, respectively.

Study Design. Experimental prospective animal study.

Methods. Vocal fold stripping was performed on 38 Sprague-Dawley rats. Vocal fold tissue was collected at five time points (3 days–2 months). Expression of HA was examined by immunohistochemistry, and messenger RNA (mRNA) expression of Has and Hyal was examined by real-time polymerase chain reaction and *in-situ* hybridization.

Results. In scarred vocal folds, expression of Has1 and Has2 increased at day 3 together with expression of HA and returned to normal at 2 weeks. At 2 months, Has3 and Hyal3 mRNA showed higher expressions than normal.

Conclusions. Expression patterns of Has and Hyal genes differed between normal, acute-scarred, and chronic-scarred vocal folds, indicating the distinct roles of each enzyme in maintaining HA. Continuous upregulation of Has genes in the acute phase may be necessary to achieve scarless healing of vocal folds.

Key Words: Vocal folds–Hyaluronic acid–Hyaluronan synthase–Hyaluronidase–Scar.

INTRODUCTION

Scarring is the single greatest cause of a poor voice after vocal fold surgery.[1] Fibrous scar tissue replaces healthy tissue in the vocal fold cover, which can irrevocably alter vocal fold function, leading to various degrees of dysphonia.[2,3] Vocal fold scarring is one of the most challenging laryngeal disorders to treat[2] because there is no consistently effective treatment.

Hyaluronic acid (HA) is a high molecular weight (10^5–10^7 Da) unbranched glycosaminoglycan, composed of repeating disaccharides (beta 1-3 D-N-acetylglucosamine, beta 1-4 D-glucuronic acid). It is a widely distributed component of the extracellular matrix (ECM) of vertebrate tissues.[4] HA is also abundant in the vocal fold lamina propria and several studies have shown that HA contributes to the viscoelastic properties of the vocal fold cover and influences tissue viscosity, playing an important role in proper vocal fold vibration.[5,6]

Recent studies[7,8] indicate that HA has important biological functions in addition to its action as a space filling material. HA is vital for embryonic development, during which it serves as a pathway for cell migration and influences cell proliferation and differentiation. For example, mice bearing a defective hyaluronan synthase (Has) 2 gene that codes for one of the three HA synthesizing enzymes, die at mid-gestation because of severe cardiovascular abnormalities.[7] This study appeared to address old questions about the biological functions of HA by providing concrete evidence that "hyaluronan is not just a goo."[8]

HA is also important in both the acute phase of wound healing and in fetal wound healing. Fetal wounds are known to repair without scarring. HA is abundant in fetal tissue and is upregulated for a longer period than in adults during the wound-healing process. HA has also been shown to improve adult wound healing.[9] Hellstrom[10] reported enhanced wound healing in tympanic membrane perforations in a rat model following the topical application of tissue-extracted HA. Hu[11] reported that three-dimensional HA grafts promoted healing and reduced scar formation in dermal wounds. In clinical trials, topical application of HA resulted in improved dermal wound healing.[12] Information on the influence of HA in vocal fold wound healing is limited and is therefore an important area of study to determine its influence on the vibration of healed vocal folds.

Animal models are essential to understanding the relationship between vocal fold scarring and HA because systematic study is not possible in humans. Several animal models of vocal fold scarring[13–16] have been examined, and findings include decreased levels of HA in the acute phase of injury.[14] We previously confirmed that HA levels are reduced in all phases of the wound healing process in a rat model[17,18]; however, little is known about the way in which HA is produced and digested in normal and scarred vocal folds. Clarification of the homeostasis of HA is critical to understand the vocal fold scarring mechanism, which ultimately will lead to improved understanding of the scarring treatment. New treatment

Accepted for publication July 31, 2014.

This study was supported by grant R01DC4428 from the National Institutes of Health/National Institute on Deafness and Other Communication Disorders. 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 the University of Wisconsin-Madison.

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Journal of Voice, Vol. 29, No. 2, pp. 133-139
0892-1997/$36.00

http://dx.doi.org/10.1016/j.jvoice.2014.07.021

strategies making use of the biological function of HA may ultimately lead to a dependable treatment for vocal fold scarring.

Thus, the purpose of this study was to clarify the homeostasis of HA in normal and scarred vocal folds. HA is known to be synthesized by three types of Has (Has1, Has2, and Has3) and to be digested by four main types of Hyaluronidase (Hyal; Hyal1, Hyal2, Hyal3, and Hyal4), in mammals. We analyzed the expression of these three Has genes and four Hyal genes using a rat model. Rats are a good model because they have a vocal fold structure which resembles that of human vocal folds with three layers in the lamina propria and a similar distribution of HA. In addition, there is an abundance of genetic information available for the rat, and information about chronic vocal fold scarring can be obtained faster because of their short life span.[17-19]

MATERIALS AND METHODS

Rat videolaryngoscopic surgery and tissue preparation

Thirty-eight male Sprague–Dawley rats (4–6 months old) were involved in the study. Rat videolaryngoscopic surgery was performed as described previously.[18] Rats were anesthetized with an intraperitoneal injection of ketamine (90 mg/kg) and xylazine (9 mg/kg). Atropine sulfate (0.05 mg/kg) was also injected intraperitoneally to reduce the secretion of saliva and sputum in the laryngeal lumen. The animals were placed on an operating platform in a near vertical position. A suspension microlaryngoscope fabricated from a 1-mm-diameter steel wire[20] was inserted through the mouth to help visualize the vocal folds. Vocal folds were monitored with a 1.9-mm-diameter telescope with an angle of 25° (Richard Wolf, Vernon Hills, IL). Using a 25-G needle and microforceps, unilateral vocal fold stripping was performed for histologic study and *in situ* hybridization and the thyroarytenoid muscle was exposed. The contralateral side was kept intact and used as a control for histologic study. For the real-time polymerase chain reaction (PCR) study, bilateral vocal fold stripping was performed and control samples were collected from untreated rats.

Larynges were harvested at five time points (3 days, 5 days, 1 week, 2 weeks, and 8 weeks) for the histological study and at three time points (3 days, 2 weeks, and 8 weeks) for the real-time PCR and *in situ* hybridization studies after creating a scar. The specimens were soaked in embedding medium (O.C.T. compound, Tissue-Tek, Kyoto, Japan), snap frozen with a combination of acetone and dry ice and stored in a deep freezer.

Immunohistochemical analysis

Ten-micrometer cryostat coronal sections of vocal folds were prepared and air dried. Double immunohistochemical staining was performed to detect HA and collagen type III in normal and scarred vocal folds, with nuclear counterstaining by TOTO-3 (Molecular probes, Eugene, OR). Sections were fixed for 1 minute at room temperature in 4% paraformaldehyde (PFA), and washed three times in phosphate-buffered saline (PBS). Sections were then blocked with 5% normal goat serum in 0.1% Triton-X in PBS for 1 hour, then incubated overnight at 4°C with mouse monoclonal anti-collagen type III antibody (1:4,000; Sigma-Aldrich, St. Louis, MO) and biotinylated HA binding protein (2.5 μg/mL; Seikagaku Co., Tokyo, Japan) with 1% normal goat serum in 0.1% Triton-X. Next day, the sections were washed in PBS and incubated for 1 hour with Cy3-conjugated anti mouse IgG (1:400; Amersham Biosciences, Piscataway, NJ), Avidin-D (1:1000, Vector Labs, Burlingame, CA), and TOTO-3 (200 nM). Finally, samples were washed three times in PBS, mounted in Vectashield and coverslipped (Vector Labs) for observation under a laser-scanning confocal microscope (Bio-Rad H600, Hercules, CA). Rat skin was used as a positive control for each staining process. Omission of the primary antibody served as a negative control.

Real-time PCR study

The microdissection technique was used to accurately collect the lamina propria from larynges. Sixty-micrometer cryostat axial sections of the vocal folds were prepared and the entire lamina propria was dissected from the sections under a microscope using 30-G needles. Tissue was collected into tubes and treated with proteinase K. Total RNA was extracted using an RNeasy Micro kit (Qiagen, Valencia, CA) and treated with RNase free DNase I (Qiagen) to digest potentially contaminated genomic DNA. Reverse transcription (RT) was performed using Superscript III (Invitrogen, Carlsbad, CA) to synthesize first-strand complementary DNA (cDNA).

Real-time PCR was performed in a 20 μL volume following the manufacturer's protocols; the reaction mix consisted of 2 μL of template cDNA, 2 μL of LightCycler DNA Master SYBR Green I (Roche Applied Science, Indianapolis, IN), 4 mM $MgCl_2$, 0.5 μM final concentration of each primer, and RNase free H_20 to 20 μL. Expression of rat Has1, Has2, Has3, Hyal1, Hyal2, Hyal3, Hyal4, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) genes was examined. Primer sets used in this study are shown in Table 1. Amplification was performed under the following conditions: heating at 95°C for 110 seconds, followed by 40 cycles of 95°C for 10 seconds, 55°C for 10 seconds, and 72°C for 20 seconds, and finally heating from 60°C to 95°C to make a melting curve. Fluorescence was detected with the Smart Cycler II system (Cepheid, Sunnyvale, CA) and accompanying software. RT(−) samples, for which reverse transcriptase was not added during RT, were also included in the PCR reaction as the negative control to check that the amplified DNA band was not the result of contamination by genomic DNA. Primer dimer production was checked by analysis of melting curves and by gel electrophoresis of PCR products. Relative quantitative analysis was performed based on the delta CT method using standard curves, and the ratios of target gene concentration to GAPDH were calculated. Target gene ratios were then normalized to the control target gene ratio. At least four animals were used for each real-time PCR experiment. Statistical analysis was performed using the Kruskal-Wallis H-test and the Student-Newman-Keuls test with a P value of 0.05 considered significant.

In situ hybridization

In situ hybridization for Has2 mRNA was performed as previously described[21] with some modifications. In brief, mouse Has2 cDNA templates were generated by PCR using primer set: