Homeostasis of hyaluronic acid in scarred rat vocal folds

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Abstract

<Introduction>

Vocal fold scarring occurs following inflammation, trauma, or surgery to the vocal folds and can result in severe dysphonia, which is difficult to treat successfully. Vocal folds are composed of various extracellular matrix (ECM) proteins including collagen, elastin, and hyaluronic acid (HA). With scarring, collagen increases and HA decreases and excessive collagen stiffens the vocal fold, and with HA reductions there is a concomitant reduction in viscosity of the vocal fold. HA reportedly is particularly important in determining the biomechanical properties of the vocal fold cover and is essential for proper vocalization. HA is also related to scarless healing, which heals without scar. Thus, for successful treatment of vocal cord scarring, it would appear critical to return the appropriate amount of HA to the structure. However, our lack of knowledge about HA synthesis and digestion makes this difficult. For example, increasing volume of HA through injection is problematic because of its short half-life. Thus, the purpose of this study is to clarify the homeostasis of HA in normal and scarred rat vocal folds. HA is synthesized by three types of HA synthase enzymes (HAS) and digested by four types of hyaluronidase enzymes(Hyal) in humans. Knowledge of hyaluronic acid synthesis and digestion in scarred vocal fold should lead to further understanding of HA homeostasis in vocal fold scarring and means for effective treatment.

<Materials and methods>

Rat laryngeal surgery and tissue preparation

Thirty eight Sprague-Dawley male rats (4-6month-old) were involved in the present study. Specially designed laryngoscope was inserted though the mouth to help visualization of vocal folds. Vocal folds were visualized by monitoring with a 1.9mm diameter telescope with an angle of 25 degree (Richard Wolf). With 25G needle, unilateral vocal fold stripping was performed and TA muscle was exposed. The other side was kept intact and was used as a control.

Larynges were harvested at 5 time points (3days, 5days, 1week, 2weeks, 8weeks) after making scar. , soaked in embedding medium (O.C.T. compound, Tissue-Tek), quickly frozen with a combination of acetone and dry ice, and kept in a deep freezer.

Histological study

Ten um cryostat coronal sections of the vocal folds were prepared and air-dried. Alcian blue stain was used to detect HA. A hyaluronidase digestion technique was used to detect HA. For the hyaluronidase digestion procedure, 50mg bovine testicular hyaluronidase (Sigma-Aldrich) was diluted

in 100ml PBS. Each section was incubated in this solution for 1 hour at 37C*. Next, the sections were stained with Alcian Blue (pH 2.5). HA was detected by comparing the sections with and without digestion. The stained area of each slide was analyzed using an image analysis system specifically developed for quantification of histological images. The lamina propria of each vocal fold was examined at original magnifications x20. Images were captured with a Nikon Eclipse E600 microscope (Nikon) and a Pixcera color camera (model PVC C). Metamorph Image Analysis Soflware (Universal Imaging) measured the density of stained regions from the lamina propria. The ratio (%) of pixels in the stained area relative to the total number of pixels in the lamina propria was provided as an indicator of each molecule's density.

RT-PCR study

Microdissection technique was used to collect the lamina propria from larynges correctly. Sixty um cryostat coronal sections of the vocal folds were prepared and lamina propria was dissected with microscope using 27G needles. Tissue was collected into tubes and treated with Proteinase K. mRNA was extracted using RNeasy Micro kit (Qiagen) and treated with RNase free DNase I (Qiagen) to digest potentially contaminated genomic DNA. Reverse transcription was performed using Superscript II (Invitrogen) to synthesize 1st strand cDNA. Polymerase chain reaction (PCR) was performed and expression of rat HAS1, HAS2, Hyal2, and Hyal3 genes were examined using a thermal cycler (RoboCycler Temperature cycler, Applied Biosystems) with the following standard protocol for most genes; one cycle of 94C for 2min, and followed by 35 cycles of 94C for 30sec, 55C for 30sec, and 68C for 2min, and one cycle of 68C for 10minuts; it was then cooled to 4C. RT(-) samples, for which reverse transcriptionase were not added during RT, was also treated with PCR reaction as a negative control samples to clarify that amplified DNA band was not made from contaminated genomic DNA.

<Results>

HA expression (Figure 1)

At days 3-7 inflammatory granulation tissue and regeneration of epithelium was seen. HA was present in the granulation tissue beneath the regenerating epithelium at days 3-7 and increased to a maximum at day 5, though it was less than that in control sides at day 3 and 7 and not significantly different from control sides at day 5. There was significantly less HA in the injured vocal folds at 2 and 8 weeks than in control vocal folds.

mRNA expression of HAS and Hyal genes

In normal vocal folds, there was expression of Hyal 2 gene. At day 3, expression of Hyal 2 gene showed increase and HAS 1 and HAS 2 also

showed increased expression. At 2 months, expression of all genes returned to the normal level.

<Discussion>

HA expression in the lamina propria decreased at all time points after injury. However, in the acute phase, HA level was relatively higher than that in the choric phase and there was an increased expression of HAS and Hyal genes. HA is reportedly related to the early phase of scarring in epidermal wound healing.. Our results suggest that HA is also important to the healing process in the early phase of vocal fold scarring. In our chronic scarred vocal fold, expression of HAS genes decreased to the normal level and the HA level was lower than that observed in the normal control. In fetal scarless wound healing, HA expression following injury continues for longer periods than that in adult wounds. Thus continuous production of HA may be necessary to restore the HA in the lamina propria and to protect vocal fold scarring suggesting treatment that could sustain higher levels of HA may be beneficial in prevention of scar formation.

Figure legend

Figure 1
The ratio of HA expressed area in scarred/control vocal fold.
* (p<0.05) and ** (p<0.01) indicates significant differences compared to the control side (1).