Hyaluronan in Human Vocal Folds in Smokers and Nonsmokers—A Histochemical Study

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Summary: Objectives/hypothesis. To study the hyaluronan occurrence in human vocal folds, with special regards to gender and smoking and to discuss the implications of findings.

Study Design. This is a descriptive/morphologic study.

Methods. Sixteen cadaveric vocal folds from eight individuals between 58 and 90 years old (six women and two men) were removed and studied morphologically. Three of the individuals had been cigarette smokers.

A direct method for hyaluronan histochemistry using a hyaluronan-binding protein probe (HABP) was used to visualize the polysaccharide. Five examiners performed an analysis of the intensities of hyaluronan staining, independently.

Results. We observed intense hyaluronan staining of the vocal folds of which those from women stained considerably stronger than those from men. Stratified squamous epithelium stained for hyaluronan in all sections, whereas respiratory epithelium only stained weakly or not at all. The highest accumulation of hyaluronan occurred subepithelially in the lamina propria, corresponding to Reinke's space. It was observed that vocal folds from smokers were more intensively stained than those from nonsmokers.

Conclusions. Hyaluronan is found in all layers of the human vocal fold. Contradictory to earlier studies, hyaluronan was visualized in squamous epithelium, where it may function as an impact protector. The occurrence of hyaluronan in smokers may have implications in the development of vocal fold inflammation and tumor initiation as hyaluronan is an important molecule in these processes.

Key Words: Vocal folds–Hyaluronan–Smoking–Cigarette–Histochemistry.

INTRODUCTION

The well-defined structures of vocal folds are exposed to many harmful events, for example, overuse, infections, and air pollution including tobacco smoke. Three well-defined layers grossly build up the vocal folds; the epithelium, the subepithelial space or lamina propria, and the muscular layers. In voice production, the glottic wave depends on the viscoelasticity of the extracellular matrix of the subepithelial layer. The extracellular tissue contains collagens and glycosaminoglycans (GAGs), one of which is hyaluronan or hyaluronic acid (HA). This molecule is an unbranched GAG consisting of repeating units of disaccharides (D-glucuronic acid and N-acetyl-D-glucosamine). HA is a major component of most extracellular matrices, especially loose connective tissue. It is synthesized on the cytosolic side of the cell membrane, and the growing chain is transported through the cell membrane to the extracellular space. In men and women who weighed 70 kg, there is approximately 15 g of HA of which one-third is found in the skin. HA also constitutes a major part of the human eye and synovial joint fluid.[1] HA synthesis is catalyzed by hyaluronan synthases of which three types with different properties have been isolated in vertebrates.[2, 3] HA is degraded by a group of enzymes called hyaluronidases or by oxidation.

An exceptionally wide range of biological functions have been attributed to HA in the body. It participates in the structure of cartilage, where it is bound to the proteoglycan aggrecan and regulates the distribution and transportation of plasma proteins in the tissue. It also regulates various cell functions such as cell proliferation, regulation of inflammation, and cell protection.[1] Furthermore, HA modulates tissue hydration and stabilizes the extracellular matrix.[4] During tissue development or damage, HA binds receptors and induces signal pathways and subsequently regulates cell motility, invasion, and proliferation.[5] In addition, HA shows a high resistance to water flow, thus forming barriers in tissues, although water can diffuse inside the molecular network. The ability of HA to coregulate cell behavior during embryonic development, healing processes, inflammation, and tumor development makes HA essential for tissue growth.

HA concentration, size, and organization have been found to change when tissues and organs differentiate and cells divide and migrate in an HA-rich extracellular matrix.[6] Furthermore, HA receptor interactions mediate important physiological processes such as signal transduction, HA internalization, and pericellular matrix assembly.[7, 8] Several HA receptors have been identified, of which CD44 is the main one[9] with involvement in many biological functions, for example, angiogenesis, tumor invasion, and metastasis.[10]

In an earlier experimental study in the rabbit, a heterogeneous distribution of HA was reported in the larynx with a main accumulation of the substance in the subepithelium of the vocal folds.[11] In the same study, there was no HA staining of the epithelium of the vocal folds.

In humans, only few studies dealing with the occurrence and distribution of HA in vocal folds have been published. The results have been somewhat contradictory. In two earlier

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studies,[12, 13] an indirect, computer-assisted image analysis was used to detect HA in male and female vocal folds, and it showed that HA was more abundant and evenly distributed in the male vocal folds compared to female vocal folds. In female vocal folds, relatively less HA was found in the superficial layers compared to deeper layers. In both sexes, intense HA staining was observed in the mid-to-deep portions of the lamina propria.

In a more recent study by Lebl et al,[14] more modern methods have been used, both to morphologically localize HA and to evaluate tissue concentration in different parts of the vocal folds in men and women. In their study, HA was found to be more abundant in the vocal folds of women compared to men, and the overall concentration was twice as high in female vocal folds. Interestingly, no HA staining was observed in the squamous epithelium. As in the earlier mentioned studies,[12, 13] intense HA staining was evident in the intermediate and deep layers of the lamina propria, and HA was also present in the connective tissue surrounding the individual vocalis muscle fibers. In women, having a higher fundamental frequency in voice production, a high amount of HA with viscoelastic properties can give adequate protection from repetitive impacts of the vibrating vocal folds. The authors suggested that large amounts of HA in the intermediate and deep layers of lamina propria—particularly in women—not only render protection but also may explain the edema formation in inflammatory conditions such as Reinke's edema.

The aim of the present study, using the same morphologic method as Lebl et al,[14] was to further ascertain the occurrence and distribution of HA in vocal folds in women and men and, in addition, to study the squamous and respiratory epithelium in detail with regards to presence of HA. Another aim was to investigate differences both in general morphology and occurrence of HA in vocal folds of smokers compared to nonsmokers.

MATERIAL AND METHODS

The patient material is summarized in Table 1.

Eight larynges (16 vocal folds) were resected within 8 hours after death. This was done according to the Norwegian autopsy regulations in 2004 (https://lovdata.no/dokument/SF/forskrift/2004-03-19-542). On gross inspection with a headlight and loupe magnifying glasses, all vocal folds looked normal. The patient's ages were between 58 and 90 years, average age 72 years. There were six women and two men. Three were cigarette smokers, two of them were women, 62 and 69 years old, respectively, and one was man, 86 years old. It was unknown for how long they had been smoking. No patient had died from causes related to diseases of the larynx or the upper airways, and no patient had been recently intubated before death.

Fixation

The cadaveric specimens were taken to fixation in a solution of 5% formalin. After a fixation period of a minimum of 7 days, the complete vocal folds were then dissected out from the arytenoid process to the anterior commissure.

Dehydration of the specimens was effected in an upgraded series of ethanol to xylene, and the specimens then embedded in paraffin wax. Serial sections were cut (4 μm thick) in vertical, coronal orientation, from two sites of the membranous vocal fold, posterior and middle/anterior parts, and mounted on glass slides.

After deparaffinization in xylene, a direct histochemistry for HA using a hyaluronan-binding protein probe (HABP) was performed as described in previous publications.[14, 15]

Control slides for HA staining were incubated with 50 units/mL of *Streptomyces* hyaluronidase (Sigma-Aldrich; Sweden AB, Stockhlm, Sweden) for 4 hours at 37°C. The hyaluronidase specifically degrades HA, thus proving the specificity of the method. The digestion experiments included controls incubated under otherwise identical conditions but without the enzyme. Photomicrographs were made by means of a Zeiss Axiophot microscope (Carl Zeiss Microscopy, D-07740 Jena, Germany).

An evaluation of the HA staining intensity of the sections was performed by three of the authors individually (L.R.O., A.E-L., and C.L.), at different occasions and at two different laboratories. The authors were not blinded to gender or smoking habits of the respective patients. In addition, two more independent morphologists were engaged to reexamine all specimens and to give their blinded evaluation of the HA staining intensities—altogether five examiners at the two laboratories.

An arbitrary grading scale was designed to semiquantify the relative HA staining intensities in the various tissue segments: 0, no staining; 1+, faint staining; 2+, intense staining; and 3+, intense staining with vacuoles.

RESULTS

HA distribution in squamous epithelium and in ciliated respiratory epithelium

The stratified squamous epithelium of the vocal folds was stained for HA in all sections. Most of them had intense staining (1+ to 2+, Table 1). Hyaluronan appeared to be more prominent in the deep layer (stratum spinosum) compared to the more superficial layer (stratum granulosum; Figure 1). The squamous epithelium of the nonsmoking male patient (Figure 2) stained more faintly for HA compared to the squamous epithelium from all other patients.

In ciliated, cylindrical respiratory epithelium HA occurred with less staining strength compared with neighboring squamous epithelium, or not at all (Figures 2 and 3). When present in the cylindrical epithelium, HA was located between or in the cells but not superficially between the cilia (Figure 4).

HA distribution in the lamina propria

In all the vocal fold specimens (except the nonsmoking male), the most striking finding was the intense color for HA subepithelially in the lamina propria, just beneath the basal membrane (Figures 1 and 3). The HA in the loose connective tissue of the lamina propria was located between the glottic surface epithelium and the vocalis muscle. However, in the deep layer facing the musculature, the HA staining was less pronounced compared to the rest of the lamina propria. There was no obvious difference in HA staining between the posterior and middle/anterior portions of the vocal fold. HA staining was