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 and 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 and 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 studies,[12 and 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 and 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.

Table 1. Semiquantitative Grading of Relative HA Staining Intensities of Vocal Fold Tissue Sections

Patient Number	Gender	Vocal Folds	Age	Smokers	Semiquantitative Grading	Staining Intensities (0, 1+, 2+, and 3+)
1.		2	69		Sq.epith	2+
					Subep.	3+
					Resp epithel	1+
					L.propria	2+
2.		2	62		Sq.epith	2+
					Subep.	3+
					Resp epithel	1+
					L.propria	2+
3.		2	58		Sq.epith	1+
					Subep.	2+
					Resp epithel	0
					L.propria	2+
4.		2	86		Sq.epith	1+
					Subep.	1+
					Resp epithel	0
					L.propria	1+
5.		2	90		Sq.epith	2+
					Subep.	2+
					Resp epithel	0
					L.propria	2+
6.		2	78		Sq.epith	2+
					Subep.	2+
					Resp epithel	1+

Patient Number	Gender	Vocal Folds	Age	Smokers	Semiquantitative Grading	Staining Intensities (0, 1+, 2+, and 3+)
					L.propria	2+
					Sq.epith	2+
7.		2	62		Subep.	2+
					Resp epithel	1+
					L.propria	1+
					Sq.epith	2+
8.		2	71		Subep.	2+
					Resp epithel	1+
					L.propria	2+
Total	Six women Two men	16		3		

Notes: 0, no staining; 1+, faint staining; 2+, intense staining; and 3+, intense staining with vacuoles.

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 and 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.

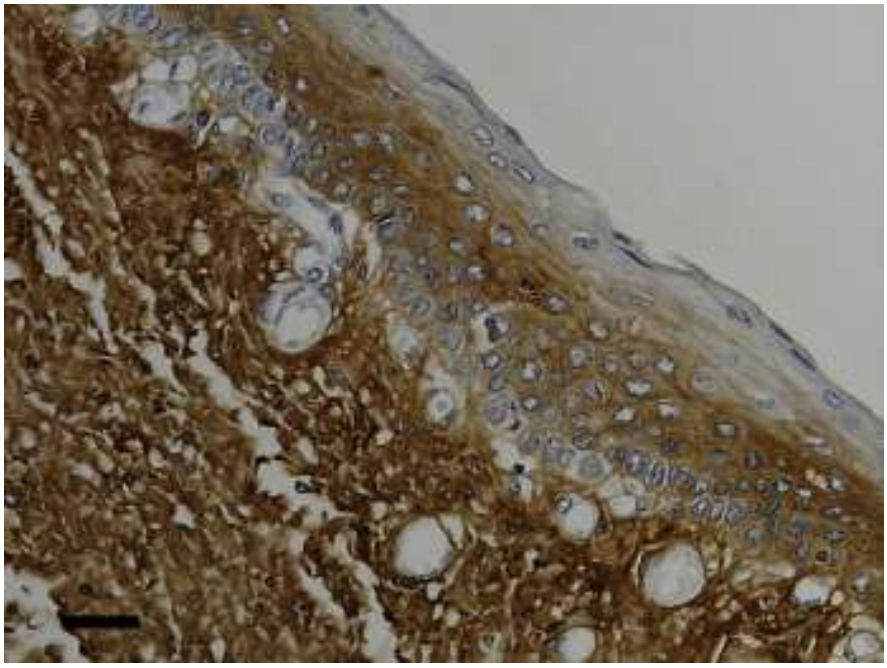

Figure 1. Color light micrographs of histological sections from various parts of human vocal folds. Bars in figure with magnification × 200 are 100 µm. Magnification × 200. Section from the middle/anterior part of the right vocal fold in patient 5, a 90-year-old female nonsmoker. Abundant HA is visible in the deep layers of the squamous epithelium and subepithelially in the lamina propria.

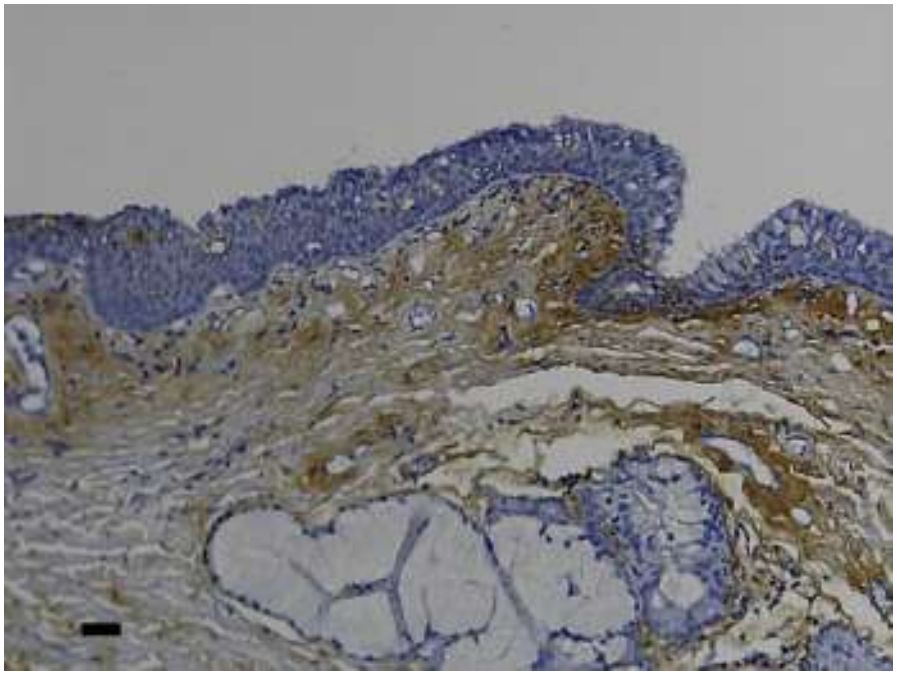

Figure 2. Bars in figure with magnification × 100 are 100 µm. Magnification × 100. Section from the middle/anterior part of the right vocal fold in patient 4, an 86-year-old male nonsmoker. The HA staining of the squamous epithelium is sparse, and no HA staining is seen in the cylindrical respiratory epithelium.

In ciliated, cylindrical respiratory epithelium HA occurred with less staining strength compared with neighboring squamous epithelium, or not at all (Figure 2 and Figure 3).

When present in the cylindrical epithelium, HA was located between or in the cells but not superficially between the cilia (Figure 4).

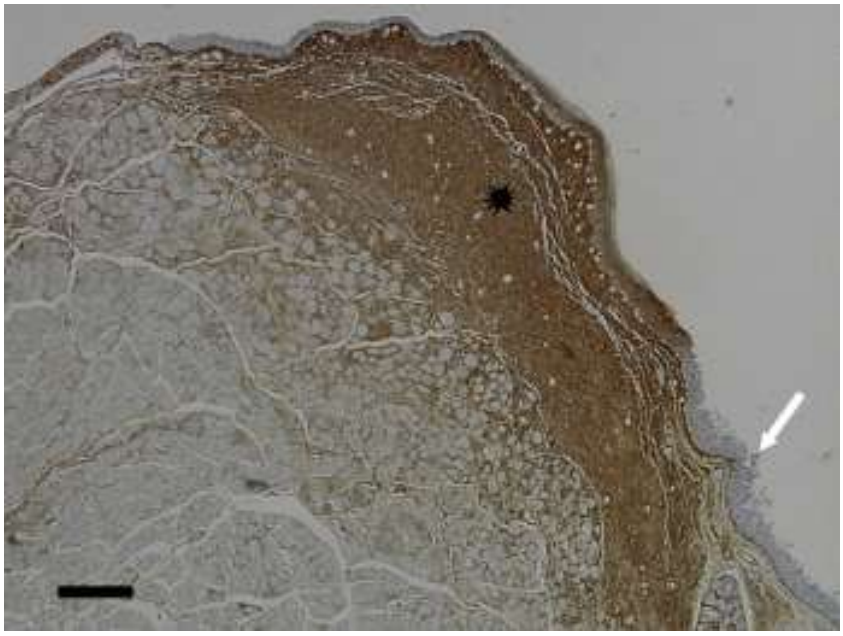

Figure 3. Bars in figure with magnification × 20 are 1000 µm. Magnification × 20. Section from the middle/anterior part of the vocal fold in patient 5, a 90-year-old female nonsmoker. Note the absence of HA staining in the cylindrical respiratory epithelium (*arrow*). In the lamina propria, there is an intense staining superficially beneath the basal lamina (*asterisk*). In the vocalis muscle, the HA staining is more pronounced in the superficial compared to the deep muscle layers.

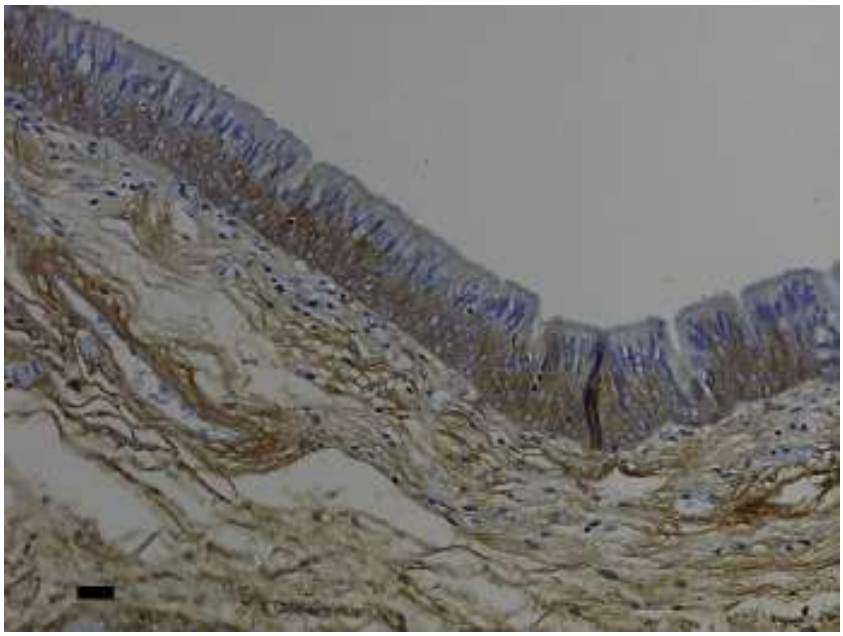

Figure 4. Bars in figure with magnification × 100 are 100 µm. Magnification × 100. Section from middle/anterior part of the left vocal fold in patient 4, an 86-year-old male nonsmoker. Ciliated, cylindrical respiratory epithelium showed limited staining strength. When present in the cylindrical epithelium, HA was located between or in the cells but not superficially between the cilia.

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 (Figure 1 and Figure 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 pronounced around the glands, whereas the luminal content of them was left unstained (Figure 5).

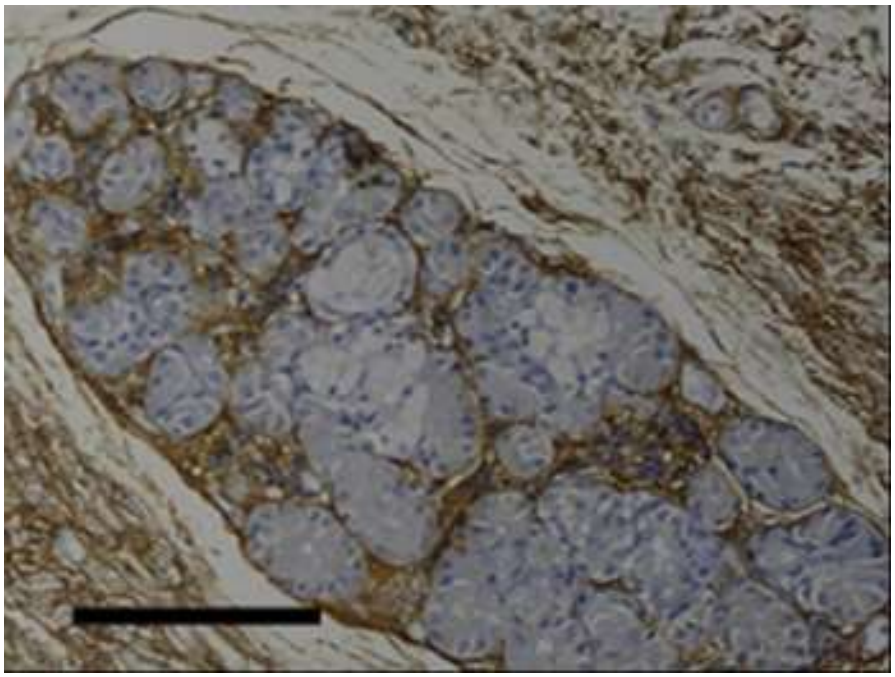

Figure 5. Bars in figure with magnification × 200 are 100 μm. Magnification × 200. Section showing glands in the vocal fold in patient 7, a 62-year-old nonsmoking woman. Note the rich HA staining in the connective tissue around the glands and the absence of HA in the luminar content of the ducts and acini.

HA distribution in the vocalis muscle (arytenoid muscle)

In the vocalis muscle of all specimens, HA was present in the connective tissue (epimysium and perimysium) enclosing the muscle fiber bundles. In some specimens, the endomysium enclosing the individual small muscle fibers also stained for HA. In all specimens, the HA staining of the vocalis muscle was more pronounced in the superficial parts with less staining intensity toward the deep muscle portions close to the thyroid cartilage (Figure 3).

Women versus men

The difference in HA staining intensity between women and men, using the semiquantitative grading scale, is summarized in Table 1 (male patients 3 and 4 vs all female

patients). There appeared to be more HA subepithelially and in the lamina propria in women compared with men.

In the histological sections, a comparison was made between patient 5 (90-year-old female nonsmoker) and patient 4 (86-year-old male nonsmoker). The male vocal folds (Figure 6) were less intensely stained for HA compared to the female vocal folds (Figure 3).

Figure 6. Bars in figure with magnification × 20 are 1000 µm. Magnification × 20. Section from the posterior left vocal fold in patient 4, an 86-year-old male nonsmoker.

In women, there was a homogenous HA staining between all layers of the vocal folds and more pronounced staining in the lamina propria, whereas in the male vocal folds, the staining was most intense subepithelially.

Smokers versus nonsmokers

To investigate the difference between smokers and nonsmokers, we choose sections from two women, both aged 62 years (patient 7, a nonsmoker and patient 2, a smoker) and the only two men in the study (patient 3, a 58-year-old male smoker and patient 4, an 86-year-old nonsmoker).

In the squamous epithelium, there were few, if any, differences in HA staining between the smokers and nonsmokers—the only difference was in the nonsmoking male patient (Figure 2) who stained weakly for HA in the epithelium compared with all other patients.

However, in the subepithelial lamina propria, the female nonsmoker had less pronounced HA staining than the female smoker (Figure 7 and Figure 8). The stronger HA staining pattern in the lamina propria of a smoker was also noticed in the male smoker compared to the nonsmoking male, but not to the same degree as in the females (Figure 6 and Figure 9).

Smokers also showed more dilated vessels just subepithelially in the lamina propria compared to the nonsmokers.

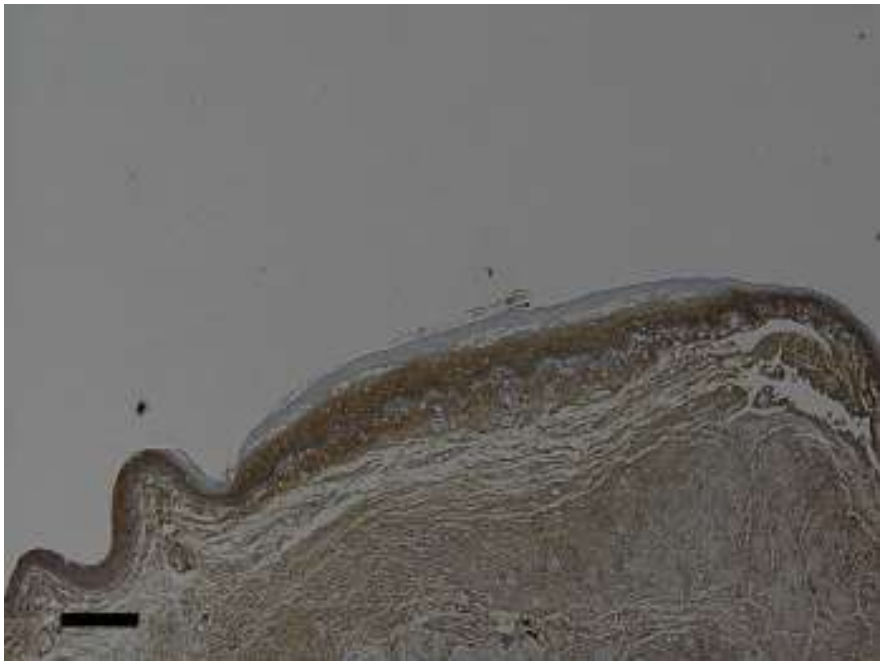

Figure 7. Bars in figures with magnification × 20 are 1000 µm. Magnification × 20. Section from the anterior left vocal fold in patient 7, a 62-year-old female nonsmoker.

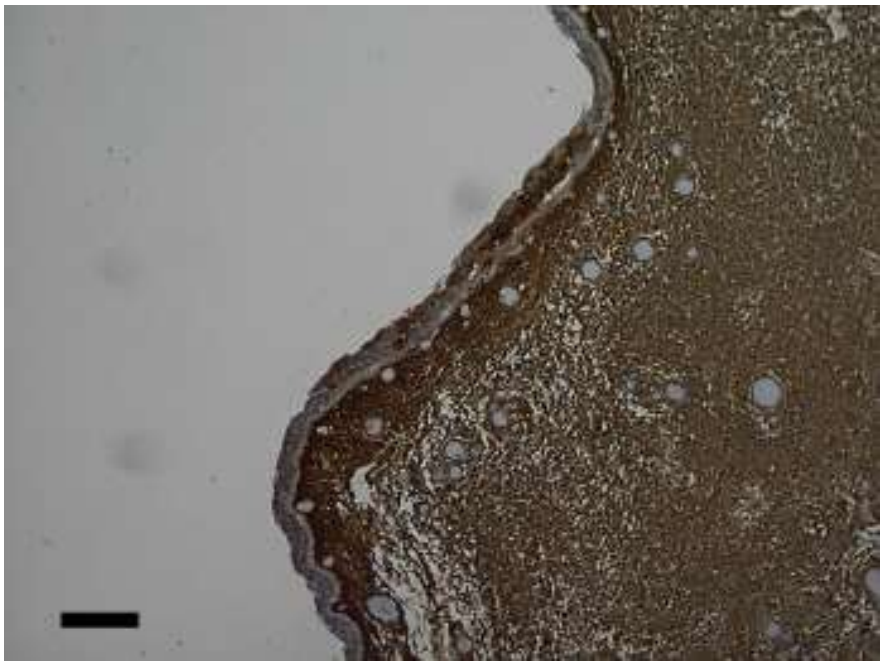

Figure 8. Bars in figures with magnification × 20 are 1000 µm. Magnification × 20. Section from the anterior left vocal fold in patient 2, a 62-year-old female smoker.

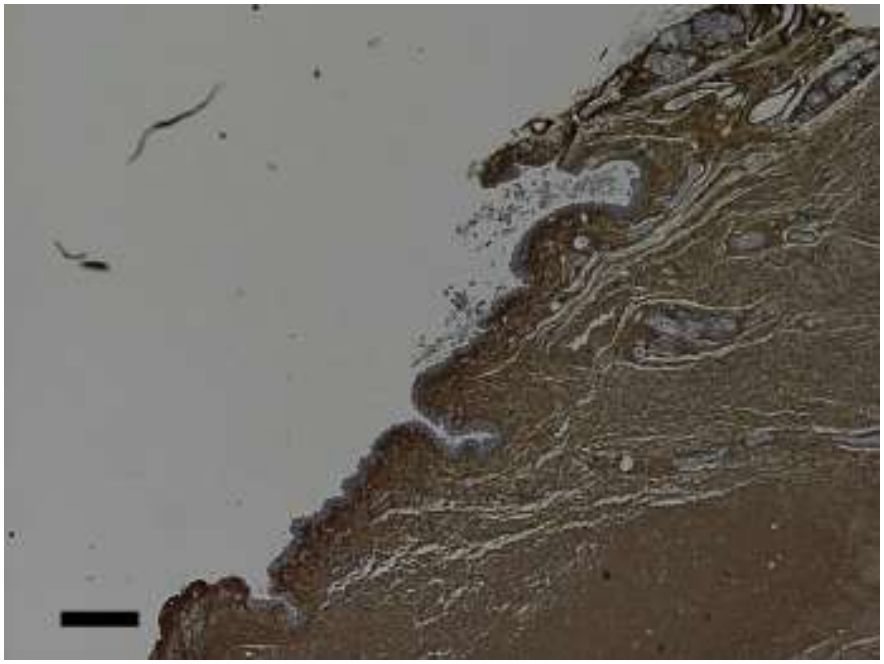

Figure 9. Bars in figures with magnification × 20 are 1000 µm. Magnification × 20. Section from the anterior left vocal fold in patient 3, a 58-year-old male smoker.

Control slides

None of the control slides that were first treated with *Streptomyces* hyaluronidase showed any staining reaction, indicating that the HABP specifically reacted with HA and not with any other GAG.

Discussion

This study showed that HA was present in all layers of the human vocal fold. Although our material was small, interesting observations could be made with regard to gender and smoking habits. From the histological sections, five experienced examiners made independent estimations of the HA staining intensities and, thus semiquantified the HA, but no true measurements of HA concentrations were made. However, several previous studies have shown that the high specificity and affinity of the HABP used with the current direct histochemical method gives a good correspondence between the staining intensities and the true amount of HA present in the tissues.[16 and 17]

Unexpectedly, we found that HA occurred in stratified squamous epithelium of vocal folds in both men and women, irrespective of smoking habits. In two earlier studies, the epithelium of the vocal folds has been studied; one in rabbits[11] and one in human adult vocal folds,[14] both using HABP for direct visualization of HA. In neither of them, HA was shown to be present in the squamous epithelium. The reason for the discrepancy between study findings can only be speculated on, but one reason could be that different fixation methods were used, as it is known that it is difficult to maintain HA in the tissue with some fixation processes.[15]

HA has been found in different urogenital epithelia giving a background to the “epithelial shape modification” theory.[16] The conclusion was that epithelia in organs which have to change their shape under varying physiological circumstances exhibit more HA between the epithelial cells because HA can facilitate cell-to-cell motility. In the vocal fold, the squamous epithelium most likely also changes in shape with the glottic wave. HA in the squamous epithelium may also act as a shock protector for the repetitive impacts between the two vocal folds in voice production. Its role in vocal fold squamous epithelium can thus be summarized as a lubricator and shock absorber allowing for epithelial movements and epithelial protection both of importance in voice production.

No prior studies have investigated HA occurrence in respiratory epithelium of vocal folds.

We saw scarce staining for HA in ciliated cylindrical respiratory epithelium and no HA between cilia in such epithelia. One explanation could be that fibroblasts exposed to mechanical forces have stronger production of extracellular matrix constituents, including HA, than fibroblasts that are not exposed to mechanical forces,[18] and respiratory epithelium takes no part in voice production. However, small accumulations of HA between cylindrical cells could facilitate smaller changes in the shape of the epithelium.

Our study showed pronounced HA staining in all layers of the lamina propria with a distinct intensive staining subepithelially. In the previous experimental study in rabbits, the lamina propria of the vocal fold also displayed a pronounced HA staining in the loose connective tissue of the subepithelium.[11] This is in contrast to a recent article[19] which states that there is no HA in the lamina propria of “unphonated” human vocal folds.

It is a well-known fact that HA is one of the main components of the lamina propria of human vocal folds.[12, 13, 14 and 20] However, previous studies report some differences between the sections. Butler et al,[13] using an indirect, computer-assisted image analysis, found a variable distribution pattern among women, notably less HA in the more superficial lamina propria, whereas in men, they showed an even distribution pattern throughout this layer. Lebl et al,[14] using a more reliable histochemical technique, revealed a clear distinction between layers with more pronounced HA staining in the intermediate and deep lamina propria in comparison with the superficial layer. Finally, Korn et al[20] found a higher HA concentration in the cover (superficial lamina propria) among women compared with men.

The only two studies that have measured HA concentrations in vocal folds[14 and 20] both came to the same conclusion that there is higher concentration in women than in men, however with some differences in distribution. The observations in our histochemical study support the findings by Lebl et al[14] and Korn et al.[20]

Previous data have confirmed the importance of HA for the biomechanical properties of the vocal fold extracellular matrix.[21] Viscosity and elasticity (stiffness) are essential in voice production because they directly affect the initiation and maintenance of phonation. High HA content leads to lower tissue viscosity. A reduction of HA in the extracellular matrix of the vocal fold may be associated with significant decrease in tissue elasticity or stiffness.[21] Women's high HA content in the lamina propria promotes lower dynamic viscosity, decreasing the required energy needed to start phonation and, an increasing tissue stiffness

favoring the production of high-frequency sounds, in accordance with the conclusions of Lebl et al.[14] In addition to regulating the vocal fold viscoelasticity, HA could act as a tissue shock absorber that may protect the vocal folds from the oscillatory trauma exerted during phonation.[22] Women's higher frequency phonation implies more tissue collisions, which demands higher HA content in the vocal fold compared to men.

HA-based injectable biomaterials are nowadays used, both in the mucosa as well as in the musculature in voice insufficiency and vocal fold paralysis. A recent Cochrane database systematic review, however, found insufficient high-quality evidence for, or against, specific injectable materials for treatment of unilateral vocal fold paralysis.[23]

In all specimens, the HA staining of the vocalis muscle was more pronounced in the superficial parts as compared with the deep muscle portions close to the thyroid cartilage. HA was located in the connective tissue (epimysium, perimysium, and endomysium) enclosing the individual muscle fiber bundles. In another study of the histochemical localization of HA in 14 different skeletal muscles,[24] it was shown that HA was particularly abundant, like dense band-like rings, around the individual muscle fibers in muscles with small fiber dimensions (eg, lateral rectus muscle of the eye and stapedial muscle of the middle ear) that has to perform fast repetitive movements. The concept is that HA in the fiber sheaths lubricates and enables these movements.[14] This might also be the demand in vocal fold muscles where precise and fast movements are extremely important.

Cigarette smoke has an irritative effect on the tissue and may induce inflammation and even tumor changes. When comparing the HA staining between smokers and nonsmokers, we observed a stronger HA staining in the lamina propria of a smoker compared to a nonsmoker, especially in women. This complies well with earlier studies in mice where it has been shown that cigarette smoke increases HA synthesis in the lungs.[25 and 26] Smokers also showed more dilated vessels subepithelially as an indirect sign of vascular dysfunction.

Cigarette smoke contains large amounts of reactive oxidation species which have HA depolymerizing properties.[27] Inflammation, tumor initiation, and angiogenesis are known to be induced by fragmented low molecular weight (LMW) HA.[28, 29 and 30] It has been suggested that HA depolymerization into LWM HA is the first step triggering the inflammatory response to cigarette smoke.[31]

Future studies should be done to determine a more detailed view of the HA metabolism in the human vocal folds both in health and disease. HA molecular weight in human vocal folds has hitherto been difficult to determine because of the small biopsies available. However, estimation of HA molecular weight in small biological samples has recently been made possible by using gas phase electrophoresis.[32 and 33] Thus, in the future, it will be possible to analyze the HA molecular weight spectra in small biopsies from vocal folds in health and disease.

Conclusion

The observations in the present study supported previous data and showed that HA is a constituent of the normal vocal fold with a variation in distribution throughout the

anatomical structure. HA is present in vocal fold squamous epithelium where it could be of importance for the sliding mobility of the mucosa and impact protection in voice production. In the lamina propria and the vocalis muscle, HA staining was prominent and it may there facilitate the viscoelastic glottic wave and lubrication of muscle fibers.

We observed more HA in female vocal folds compared with male vocal folds, ascertaining findings from other recent studies. It is feasible that HA favors the production of a high-frequency voice in women, works as a shock absorber, and protects the vocal folds from mechanical impacts.

It seems that smoking might influence HA in the vocal folds. This has not been shown before and may have implications in the development of vocal fold inflammation, creation of edema, and tumor initiation as HA is involved in these processes.

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