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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.

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Keywords

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 up-regulated 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

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 cover-slipped (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:

5′-ATGCATTGTGAGAGGTTTCTATGTGTCCTG-3′ (forward) and 5′-TACTGTATAGCCCCTTGAGGAGCTAAGGTG-3′ (reverse). A 1743 bp mouse Has2 cDNA, including the entire coding region, was subcloned into the pGEM-T Easy vector (Promega, Madison, WI) and used as the template to generate digoxigenin (DIG)-labeled RNA probes by *in vitro* transcription. Templates were generated by linearization with SalI for antisense probes and with NcoI for sense probes. Antisense probes were synthesized by run-off transcription from the T7 promoter with DIG-UTP using an RNA labeling kit (Roche Applied Science). Meanwhile, DIG-labeled sense probes, synthesized by run-off transcription from the SP6 promoter, were used as negative control probes. Ten-micrometer cryostat coronal sections of vocal folds were prepared and air dried. Sections were fixed in 4% PFA for 30 minutes and rinsed twice with 0.1 M PBS (pH 7.5) containing 0.1% Tween 20 for 5 minutes. Hybridization was performed at 65°C overnight in hybridization buffer (50% formamide, 1% dextran sulfate, 5 × standard saline citrate (SSC), 50 μg/mL heparin, 50 μg/mL yeast transfer RNA) containing DIG-labeled RNA probes. The sections were washed in 5 × SSC at 65°C for 30 minutes, three times in 2 × SSC at 65°C for 30 minutes, and three more times with Tris-buffered saline with Tween 20. The probes were visualized using alkaline-phosphatase-coupled anti-DIG antibodies (Roche Applied Science), which were reacted with nitroblue-tetrazolium-chloride and 5-bromo-4-chlor-indolyl-phosphate substrates for the color reaction. Rat whole embryo (E16.5) sections were used as positive control specimens.

RESULTS

HA expression

Immunohistochemical staining showed that, in the normal rat vocal fold, HA was present in the intermediate and deep layers of the lamina propria, whereas collagen type III was distributed sparsely throughout the whole lamina propria (Figure 1). These distribution patterns were similar to those reported for the human vocal folds.

At day 3, inflammatory granulation tissue and regeneration of epithelium were seen. HAwas also present in the granulation tissue beneath the regenerating epithelium at days 5 and 7.[17] By 2 weeks, collagen type III had become dominant, whereas less HA could be detected. At 2 months, HA remained at the same level and collagen type III remained dominant in the regenerated lamina propria.

Has mRNA expression in normal and scarred vocal folds

Changes in Has gene expression after vocal fold surgery are shown in Figure 2. The values on the *y*-axis represent the normalized mRNA expression ratios of investigated genes. At day 3, both Has1 and Has2 expressions increased significantly. In particular, expression of Has2 increased almost ninefold. At 2 weeks after surgery, expressions of both Has1 and Has2 returned to control levels and remained at the same level at 2 months. Has3 expression showed the opposite pattern to Has1 and Has2, with a significant decrease at day 3, but an increase at 2 weeks and thereafter and was significantly higher than control and increased at 2 months after surgery.

Hyal mRNA expression in normal and scarred vocal folds

The change of Hyal gene expression after vocal fold surgery is shown in Figure 3. The values on the *y*-axis represent the normalized mRNA expression ratios of the investigated genes. Hyal1, Hyal2, and Hyal4 did not change significantly. However, Hyal3 expression increased at 2 months, when it was significantly higher than at the other time points examined.

In situ hybridization of Has2

In situ hybridization detected little Has2 signal in the normal vocal fold (Figure 4). In contrast, at day 3 after surgery, strong expression of Has2 was observed in all areas of regenerating tissue. At 2 months, little expression was seen anywhere in the lamina propria.

DISCUSSION

HA in scarred vocal folds

In cutaneous wound healing, HA expression appears early and modulates numerous stages of wound repair.[9] In the early stages of repair, HA levels increase and cell migration occurs, restoring cellular continuity. HA provides a temporary matrix for the migration of inflammatory cells and proliferation of fibroblasts in the connective tissue. HA has been found to be abundant around the migrating cells of wounded human oral epithelium and among cells surrounding the wound.[22] Later, when HA concentration falls and sulfated glycosaminoglycan concentrations are high, cell differentiation, collagen production, and tissue organization occur.[9] Biochemical assays on experimental dermal wounds also indicate that HA reaches its peak concentration 3 days after a wound is created and start to decline before levels of proteoglycans and collagens reach their peak.[23]

In the vocal folds, Thibeault[14] reported in a rabbit study that HA levels after injury decrease immediately after wound creation and increase transiently at day 5 such that the level of HA does not differ significantly from that found in normal vocal folds. Our previous studies in rats[17] have also shown that a transient peak of HA concentration occurs in scarred vocal folds at day 3 and day 5 after wound creation and that this expression subsequently decreases. In the present study, we confirmed the presence of HA in granulation tissue beneath the regenerating epithelium at day 3. These results are consistent with those reported in other regions of the body[22,23] suggesting that HA plays an important role in the vocal fold wound healing process as well as in other tissues and organs.

Has and Hyal expression in normal and scarred vocal folds

Has protein is located in the cell membrane and produces HA in the ECM. In mammals, Has genes have three subtypes: Has1, Has2, and Has3. Has2 and 3 have stronger enzymatic activity than Has1. Has1 and Has2 produce longer HA chains, resulting in higher molecular weight HA.[24] Has2 is essential for embryonic development, as exemplified by the death of Has2 deficient mice.[7] On the other hand, uninjured double-knockout mice lacking both Has1 and Has3 are phenotypically normal.[25] All three Has enzymes have been suggested to play roles in cutaneous responses after injury.[25] In excisional wound healing experiments, faster wound closure, exaggerated neutrophil recruitment, a decrease in epidermal and dermal HA,

and a compensatory increase in dermal Has2 expression have been observed in Has1/3 double-knockout skin.[25] In the larynx, Thi-beault[26] reported Has2 expression in normal vocal folds.

In humans, six paralogous Hya1 genes are known to be expressed; Hyal1, Hyal2, Hyal3, Hyal4, HYALP1, and PH-20 (SPAM1). However, less is known about Hyal than Has. In this study, we only examined the expression of Hyal1, 2, 3, and 4, because HYALP1 is a pseudogene with no significant enzymatic function and PH-20, which is involved in fertilization, has been investigated in greater detail, and is known to be expressed in a limited fashion in testis.[27] Hyal1 is located in the lysosome, and human plasma and urine are both known to contain Hyal1. Hyal2 is present in many tissues with the notable exception of the brain where it is expressed only during embryonic development and for a short time after birth. Hyal2 is located in lysosomes as well as on the plasma membrane via a glycosylphosphatidylinositol anchor, suggesting that Hyal2 functions in tissue turnover and remodeling.[27] Indeed, Hyal2 has been shown to be involved in HA turnover during the early phase of lung injury and growth of astrocytomas in mice.[27] Hyal2 is also expressed much more strongly than Hyal1 in lung tissue.[28] Hyal3 is also present in many different tissues, particularly in testis, but no detailed biochemical studies have been performed. Little is known about Hyal4.

Several studies[28–33] have focused on Has gene expression in wound healing *in vivo* and *in vitro*. In our study, Has1 mRNA and Has2 mRNA expression increased at day 3 but these expression patterns returned to normal after 2 weeks. Increased Has1 expression during the acute phase of injury has been reported in dermal fibroblasts[30] and oral mucosal epithelial cells[31] *in vitro* and increased Has2 during the acute phase has also been reported in ischemic injury to the kidney *in vivo*[29] and in epidermal keratinocytes,[33] lung,[32] dermal and oral mucosal fibroblasts[31] *in vitro*. Our results indicate that both Has1 and Has2 are involved in the acute phase wound healing process, being upregulated immediately after injury to increase HA levels in the scarred vocal folds. Interestingly, Has3 mRNA in our study showed patterns that were the opposite of the other two subtypes, showing less expression in the acute phase and increased expression in the chronic phase. Although little is known about the functional role of Has3 in wound healing, Li[28] reported the weak expression of Has3 in chronic lung injury after irradiation. In the chronic phase of wound healing, tissue remodeling replaces old ECM structures with new components. Expression of Has3 may be related to the remodeling process of HA in the chronic phase of wound healing.

As for the expression of Hyal after injury, our study showed no significant difference in the expression of Hyal1, Hyal2, or Hyal4 mRNA in scarred vocal folds versus those in normal vocal folds. In a lung irradiation injury, Li[28] found induced expression of Hyal1 and Hyal2 which peaked at 4 weeks after irradiation, followed by marked decreases to levels below the constitutive expression seen in lung tissues from normal rats. This discrepancy in the expression pattern may be due to either the method of injury or the tissue type. Our study also showed increased Hyal3 mRNA expression at 2 months after injury. Increased expression of Has3 mRNA and Hyal3 mRNA in the chronic vocal fold scar may suggest that Has3 and Hyal3 are related to the remodeling process of HA in a chronic wound.

In summary, the expression patterns of Has and Hyal genes differ between normal, acute-scarred, and chronic-scarred vocal folds. Although the roles of each subtype in living tissue remain unclear, there seems to be a distinct role for each enzyme in maintaining HA in the vocal folds. Further studies will be necessary to examine the functions of Has and Hyal genes in the vocal fold tissue.

Scarless healing

The wound healing process can occur in two ways. One is by regeneration, which is illustrated by replacement of the lost tissue by identical new tissue. The other is repair, which results in the formation of scar tissue with denser collagen. In contrast to adult wound repair, early gestational fetal wounds heal by a process of regeneration in which the epidermal and dermal layers are perfectly reconstituted without scar formation.[9] Knowledge of this fetal wound healing mechanism seems likely to be beneficial in achieving scarless healing of the vocal folds. There are several notable contrasts in the course of fetal versus adult wound healing.[34] Fetal wounds close faster, show little or no inflammatory response, and exhibit a different profile of cytokine expression and higher levels of HA.[34] Several studies suggest that HA plays a crucial role in fetal wound healing.[34] Indeed, it has been shown that a high and persistent HA concentration in the fetal wound may influence the nature of the collagen fibrils formed, thus resulting in a prewound pattern of collagen deposition.[35] Recently, it has been reported that mice deficient in Hoxb13, a transcription factor which is expressed in fetal tissue, exhibit abundant HA in skin tissue and show rapid dermal wound healing with less scarring.[34] In the HA-rich matrix seen in fetal wounds, HA may reduce collagen deposition, enhance remodeling, and contribute to reduced scarring.[36,37] Studies on fetal lambs showed that HA increased rapidly in fetal wound fluid and remained elevated for 3 weeks. It increased more slowly in adult wounds, with a peak at 3 days followed by a sharp decrease to zero at 5 days.[4,37] In our study, we found increased expression of Has1 mRNA and Has2 mRNA with an increase of HA level in the acute phase of injury. However, the increased expression of Has genes returned to normal at 2 weeks. We interpret this to mean that continuous upregulation of Has gene expression in injured tissue may be necessary to reduce collagen deposition, enhance remodeling and contribute to achieving scarless wound repair.

CONCLUSIONS

In our study, we examined the expression of all subtypes of Has and Hyal mRNA in normal and scarred vocal folds. In scarred vocal folds, Has1 mRNA and Has2 mRNA expression increased at day 3, together with similarly increased expression of HA. Subsequently, expression of all Has genes returned to normal at 2 weeks. Continuous upregulation of Has genes may be necessary to achieve scarless healing of vocal folds similar to that observed in the fetus. At 2 months after wounding, Has3 mRNA and Hyal3 mRNA both showed higher expression than normal. Thus, Has3 and Hyal3 may be related to the remodeling process of HA in chronic vocal fold scarring. The expression patterns of Has and Hyal genes differed between normal, acute-scarred, and chronic-scarred vocal folds. Although the roles of each Has and Hyal subtype in a living tissue are still unclear, there seems to be a distinct role for each enzyme in maintaining HA in the vocal folds. Understanding the homeostasis of HA in

scarred vocal fold tissue should enable us to develop new and better treatments for vocal fold scarring.

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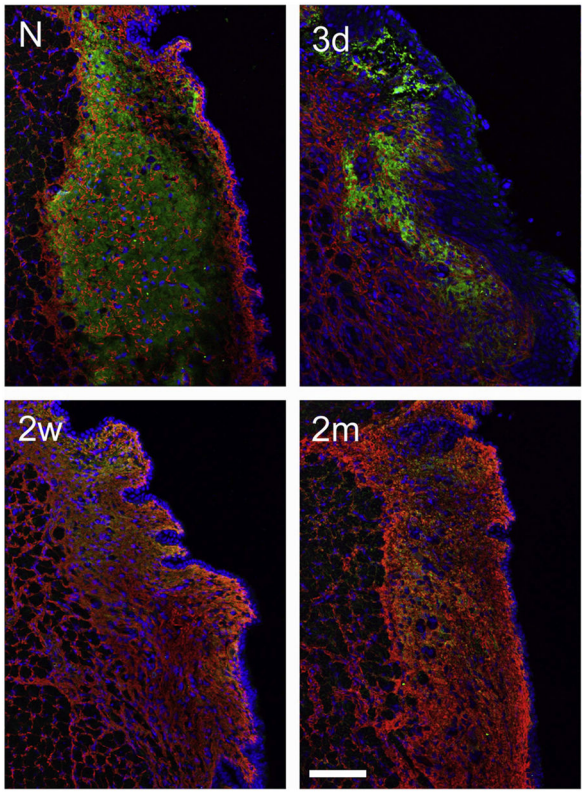

FIGURE 1.

Immunohistochemical staining of HA and collagen type III in normal and scarred vocal folds. In normal vocal folds, HA (green) is dominant, located in the middle, and deep layer of the lamina propria, whereas collagen type III (red) is distributed sparsely throughout the entire lamina propria. At day 3, HA is present in the regenerating tissue beneath the epithelium. At 2 weeks and 2 months, collagen type III is dominant and there is less HA in the vocal fold. Blue, nucleus; N, normal; 3d, 3 days; 2w, 2 weeks; 2m, 2 months. Scale bar indicates 100 μm.

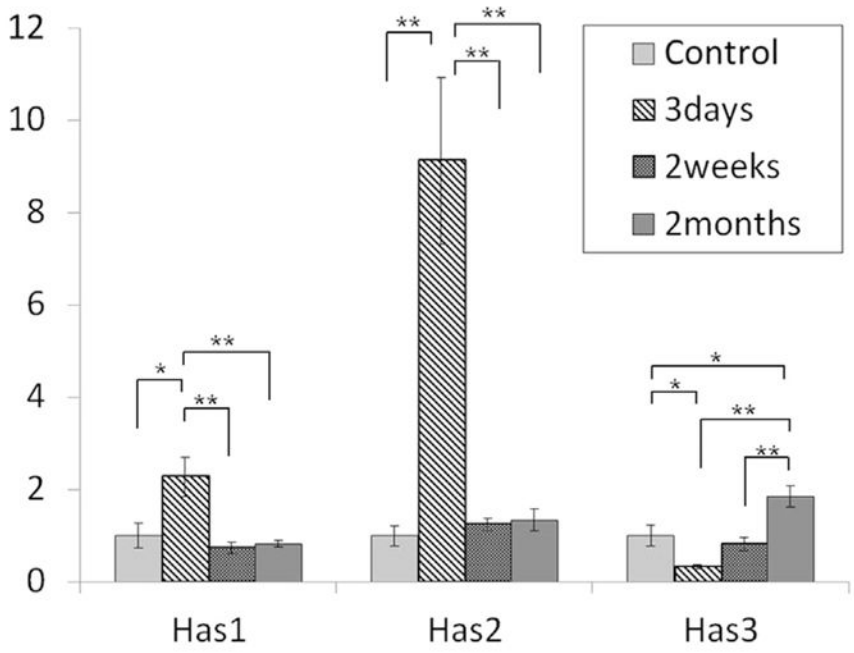

FIGURE 2.

Mean values of Has mRNA expression obtained by real-time PCR in scarred vocal folds. *$P < 0.05$ and **$P < 0.01$ indicate significant differences compared with normal controls. The value on the *y*-axis represents the normalized mRNA expression ratios of investigated genes. Error bars indicate standard error.

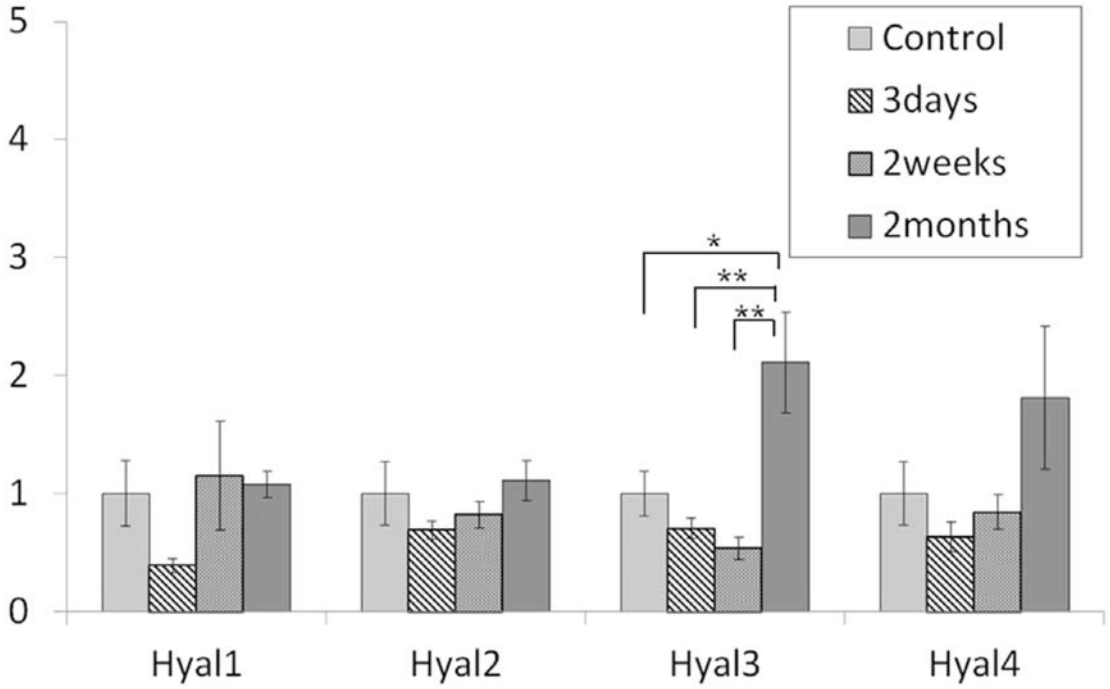

FIGURE 3.

Mean values of Hyal mRNA expression obtained by real-time PCR in scarred vocal folds. **P* < 0.05 and ***P* < 0.01 indicate significant differences compared with normal controls. The value on the *y*-axis represents the normalized mRNA expression ratios of investigated genes. Error bars indicate standard error.

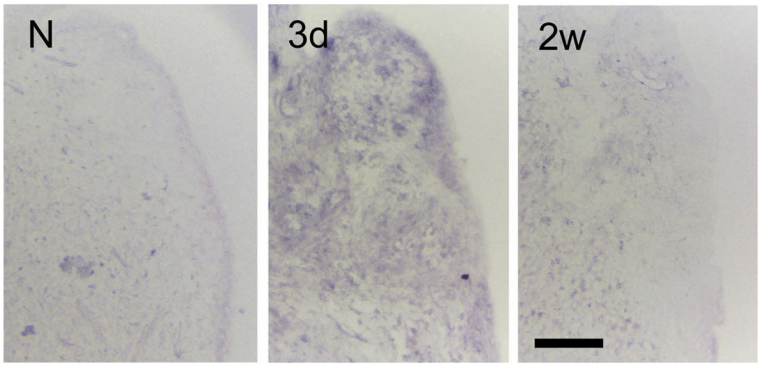

FIGURE 4.

Distribution of Has2 mRNA in normal and scarred vocal folds. In normal vocal folds, *in situ* hybridization detects little signal of Has2. At day3, Has2 mRNA is expressed in all injured areas but expression returns to normal at 2 weeks. Scale bar indicates 60 μm.

TABLE 1

Primer Information

Gene Product	Primer Sequence	Product Size
Has1	5′-TAGGTGCTGTTGGAGGAGATGTGA-3′ 5′-AAGCTCGCTCCACATTGAAGGCTA-3′	105 bp
Has2	5′-ACTGGGCAGAAGCGTGGATTATGT-3′ 5′-AACACCTCCAACCATCGGGTCTTCTT-3′	115 bp
Has3	5′-TAGTGGATGGCAATCGCCAGGAAGAT-3′ 5′-TTCACCCGCCTCATGGAAATTGCT-3′	119 bp
Hyal1	5′-ACCAAGGAGTCATGCCAAGCCATT-3′ 5′-AGAACACAGAGCTTCGCTGCACAA-3′	108 bp
Hyal2	5′-ATCCTCCAAACACAGCCGCAACTT-3′ 5′-ATGTGGGACGCGTGAAGACATACA-3′	116 bp
Hyal3	5′-AGGAACAGCTCCACAAAGCCCAAA-3′ 5′-TTATGCCAGCCATTGCCACAGACT-3′	145 bp
Hyal4	5′-CACTTCTCACAATTTCGGGTGCGTGAA-3′ 5′-TGTAGCCCAGCCGTGTGTAGACAAATA-3′	100 bp
GAPDH	5′-ATGGTGAAGGTCGGTGTGAA-3′ 5′-TCCCGTTGATGACAAGCTTC-3′	208 bp

Abbreviations: Has, hyaluronan synthase; Hya, hyaluronidase; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.