

## Hylan B Gel Restores Structure and Function to Laser-Ablated Canine Vocal Folds

Babak Jahan-Parwar, MD; Dinesh K. Chhetri, MD; Ming Ye, MD;  
Steven Hart, MD; Gerald S. Berke, MD

**Objectives:** We evaluated cross-linked hyaluronic acid (hylan B gel) as a scaffold for tissue regeneration and mucosal wave restoration in carbon dioxide laser-ablated canine vocal folds.

**Methods:** Five beagles underwent stroboscopy before ablation of the left vocal fold with a carbon dioxide laser. Four weeks later, stroboscopy was repeated before and after submucosal injection of hylan B gel into the left vocal fold of 4 animals and of saline solution in 1 animal. Stroboscopy was repeated 12 weeks later, and histologic analysis was performed.

**Results:** Four weeks after laser ablation, all animals had soft tissue defects and absence of mucosal waves. Hylan B injection restored mucosal waves, and saline injection did not. Twelve weeks after injection, hylan B-injected larynges had tissue regeneration and mucosal waves, and the saline-injected larynx had neither. Histology showed regenerated lamina propria with residual foci of hylan B in the hylan B-injected larynges and dense submucosal scar in the saline-injected animal.

**Conclusions:** Submucosal hylan B gel injection in laser-ablated canine vocal folds restored tissue volume and mucosal waves and facilitated functional tissue regeneration over 12 weeks. Hylan B gel may have utility as a soft tissue scaffold for rehabilitation of phonatory function in vocal folds with lamina propria defects.

**Key Words:** hyaluronic acid, hylan B, lamina propria, larynx, laser, tissue engineering, voice.

### INTRODUCTION

Laryngologists are frequently called upon to treat diseases resulting from loss of vocal fold lamina propria. Lamina propria defects can be due to multiple causes, including atrophy, trauma, scarring, and endoscopic laser resection for vocal fold carcinoma. An ideal method for reconstruction of lamina propria would be an implant that has viscoelastic properties similar to those of native lamina propria, allows for ultimate replacement by natural lamina propria, and is nontoxic, immunologically inert, and easily placed.

Hyaluronic acid is an integral extracellular matrix protein in both the human and canine vocal fold laminae propriae. It provides structural support and contributes to the viscoelastic properties of the vocal fold lamina propria. Hyaluronic acid has been shown to have viscoelastic properties similar to those of natural vocal fold lamina propria.<sup>1</sup> Rabbit studies show that hyaluronic acid injected into rabbit vocal folds undergoes rapid degradation, how-

ever.<sup>2</sup> Cross-linked hyaluronic acid (hylan B gel) is a derivative of natural hyaluronic acid that has enhanced rheological properties, has resistance to degradation, and promotes connective tissue ingrowth into injected sites. Hylan B gel has been proposed as an augmentation material for vocal fold insufficiency.<sup>3</sup>

Large defects of the vocal fold, such as those following endoscopic laser resection for glottic carcinoma, involve multiple layers of lamina propria. The layers of the human and canine vocal fold laminae propriae have differing compositions of ground substance, collagen, and elastin, as well as differing predominances of cellular elements.<sup>4,5</sup> Regeneration of complete lamina propria defects requires heterogeneous cellular infiltration and heterogeneous production of extracellular matrix, depending upon the depth from the mucosal surface. Vocal fold fibroblasts have been shown to vary the type of extracellular matrix produced, depending upon their environment.<sup>6</sup> Provision of an artificial soft tissue scaffold

From the Division of Head and Neck Surgery, University of California–Los Angeles Medical Center, Los Angeles, California. Dr Jahan-Parwar is currently at the Department of Head and Neck Surgery, Southern California Permanente Medical Group, Baldwin Park, California. Funding was provided by the Division of Head and Neck Surgery, University of California–Los Angeles Medical Center. 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 California–Los Angeles Medical Center.

Presented at the meeting of the American Laryngological Association, Phoenix, Arizona, April 30–May 1, 2004.

**Correspondence:** Gerald S. Berke, MD, Division of Head and Neck Surgery, 200 Medical Plaza, Suite 550, Los Angeles, CA 90095.

fold with viscoelastic properties similar to those of native vocal fold that facilitates fibroblast ingrowth and allows for a graded pressure environment for infiltrating fibroblasts may enhance soft tissue regeneration that reconstitutes the layered cellular and extracellular structures and hence the function of the native vocal fold. This study investigates the efficacy of hylan B gel as a scaffold for regeneration of vocal fold soft tissue volume and restoration of lamina propria function after ablation with the carbon dioxide laser.

## MATERIALS AND METHODS

This study was approved by the Animal Research Committee of the University of California–Los Angeles Office for Protection of Research Subjects. Animal care, housing, and surgery were conducted according to committee guidelines. Five adult beagles weighing approximately 10 kg were used for this experiment. Laryngeal stroboscopy was performed as previously described<sup>7</sup> (brief synopsis below). Airflow was titrated to the lowest level necessary to induce a mucosal wave. Three procedures were performed, as described below.

*Laryngeal Examination and Lamina Propria Ablation.* After induction of anesthesia with intravenous thiopental sodium and inhalational isoflurane, each animal was intubated endotracheally. The anterior neck skin was shaved, prepared, and draped in the usual sterile fashion. A midline vertical incision was made to expose the laryngotracheal complex. Stimulating electrodes were placed on the superior and recurrent laryngeal nerves. A low tracheotomy was made and a 6.5-mm endotracheal tube was placed for ventilation. The ventilation circuit was switched to the tracheal tube, and the oral endotracheal tube was removed. A second tracheotomy was placed superior to the first tracheotomy, and an endotracheal tube was placed directed superiorly for passage of air across the vocal folds. Videolaryngoscopy and stroboscopy were performed to record and assess the motion and quality of the vocal fold mucosal waves.

After laryngoscopy and stroboscopy, the superior endotracheal tube was removed and a carbon dioxide laser was used to ablate the mucosa and lamina propria of the left vocal fold. The animal was reintubated orally, and the lower neck endotracheal tube was removed. The tracheal and skin incisions were closed with 3-0 chromic sutures and 3-0 Dexon sutures, respectively. Once the animal was breathing spontaneously, the oral endotracheal tube was removed and the animal was allowed to recover.

*Laryngeal Examination and Lamina Propria In-*

*jection Performed Four Weeks After Lamina Propria Ablation.* Anesthesia was induced, superior and inferior tracheotomies were performed, and videolaryngoscopy and stroboscopy were performed as described above.

After laryngoscopy and stroboscopy, the left vocal folds of 4 animals were injected submucosally with 1 mL of cross-linked hyaluronic acid (hylan B gel, Genzyme Corporation, Cambridge, Massachusetts). The left vocal fold of the control animal was injected with 1 mL of normal saline solution. The animals were reintubated orally, and the lower neck endotracheal tube was removed. The tracheal and skin incisions were closed with 3-0 chromic sutures and 3-0 Dexon sutures, respectively. Once the animals were breathing spontaneously, the oral endotracheal tube was removed and the animals were allowed to recover.

*Laryngeal Examination and Euthanasia Performed Twelve Weeks After Lamina Propria Injection.* Anesthesia was induced, superior and inferior tracheotomies were performed, and videolaryngoscopy and stroboscopy were performed as described above.

After laryngoscopy and stroboscopy, the animals were euthanized with intravenous Eutha-six solution (Western Medical Supply, Arcadia, California). The larynges were harvested for histologic examination.

The injected vocal folds from each animal were submitted in toto for routine hematoxylin and eosin staining. Representative sections of the midmembranous noninjected vocal folds of each animal were submitted. Blinded slide review by a pathologist was performed to assess for the presence of lamina propria or scar in the submucosal location and for the presence of residual hylan B. Videostroboscopic results were reviewed independently by 2 surgeons (B.J.-P., D.K.C.) familiar with canine stroboscopy and were rated as described in the Table.

## RESULTS

All animals had symmetric mucosal waves on baseline phonation and normal-sounding barks. After lamina propria ablation, none of the animals were able to bark because of extreme breathiness.

Upon phonation 4 weeks after lamina propria ablation, all animals showed full mucosalization of the left vocal fold remnant with a large glottic gap present. There was some anterior subluxation of the left arytenoid cartilage in all animals. None of the animals could be phonated because of the presence of the glottic gap. All animals still were unable to bark. The

## STROBOSCOPY RATINGS AND HISTOLOGY

| <i>Animal</i>  | <i>Baseline<br/>Phonation</i> | <i>4-Week<br/>Phonation<br/>Before<br/>Injection</i> | <i>4-Week<br/>Phonation<br/>After<br/>Injection</i> | <i>16-Week<br/>Phonation</i> | <i>Histology of Injected Fold</i> |
|----------------|-------------------------------|--|---|------------------------------|-----------------------------------|
| Control        | 5                             | 1  | 1   | 1                            | Scar                              |
| Experimental 1 | 5                             | 1  | 3   | 4                            | Lamina propria with focal hylan B |
| Experimental 2 | 5                             | 1  | 3   | 4                            | Lamina propria with focal hylan B |
| Experimental 3 | 5                             | 1  | 3   | 4                            | Lamina propria with focal hylan B |
| Experimental 4 | 5                             | 1  | 3   | 4                            | Lamina propria with focal hylan B |

Stroboscopy rating scale: 1 — no mucosal waves present in injected fold; 2 — minimal vibration of injected fold; 3 — full wave excursion in injected fold with gross asymmetry; 4 — full wave excursion in injected fold with near-symmetry; 5 — full wave excursion in injected fold with symmetry.

control animal had no mucosal wave on stroboscopy after submucosal saline injection. Mucosal waves were obtained on stroboscopy in all 4 experimental animals after hylan B injection. In 3 of the experimental animals, mucosal waves were obtained with recurrent laryngeal nerve stimulation alone. These 3 animals had restoration of bark volume after injection. In 1 of the experimental animals, the left neocord was inferiorly displaced relative to the right vocal fold, impairing symmetric contact. Simultaneous stimulation of the superior and recurrent laryngeal nerves in this animal raised the vertical position of the left neocord, allowing its apposition to the right vocal fold and formation of a mucosal wave. After injection, this animal had increased bark volume with some slight persistent breathiness. The mucosal waves of all of the experimental animals had full excursion, but were grossly asymmetric.

At 12 weeks after lamina propria injection, the control animal showed minimal left vocal fold regeneration, but a persistent large glottic gap prevented phonation. In all of the experimental animals, there was left vocal fold regeneration with nearly symmetric mucosal waves present on stroboscopy. In 3 of the 4 experimental animals, mucosal waves were obtainable with recurrent laryngeal stimulation alone. These animals retained normal bark volume. The fourth animal required simultaneous superior and recurrent laryngeal nerve stimulation to obtain a mucosal wave. This animal had greater bark volume than before injection, but still had slight breathiness. The interobserver reliability of the mucosal wave ratings was 100%. Histologic evaluation of the control larynx revealed mild chronic inflammation and submucosal scar tissue without lamina propria regeneration (see Figure, C). Histologic evaluation of the experimental larynges revealed mild chronic inflammation and lamina propria regeneration (see Figure, B). Isolated foci of residual hylan B gel were visible in the lamina propria of the experimental animals. There was no associated inflammation or foreign body reaction associated with the residual

hylan B.

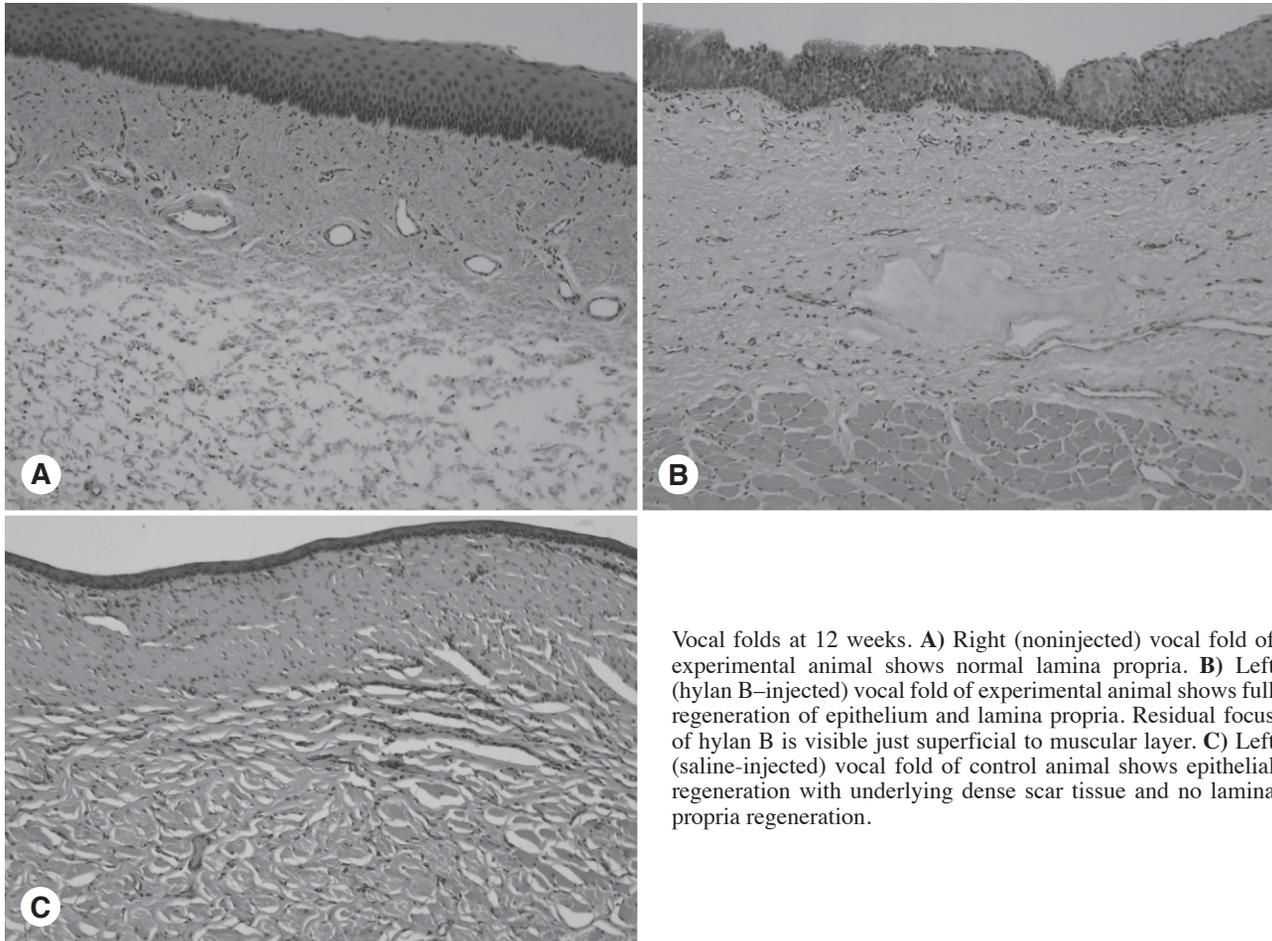
## DISCUSSION

Tissue engineering employs principles from cell transplantation, materials science, and engineering to develop biological substitutes to restore and maintain normal function. Tissue engineering may involve extracellular matrices that orient or direct new tissue growth, or the use of matrices with cells.<sup>8</sup>

Tissue engineering principles have been used to regenerate vocal fold structure in dogs. Kanemaru et al<sup>9</sup> used autologous mesenchymal stem cells derived from bone marrow in an atelocollagen matrix derived from porcine skin to promote regeneration of cauterized canine vocal folds. Restoration of tissue volume in the larynx may not be sufficient for restoration of function. Restoration of the layered structure of the vocal fold lamina propria is critical to the mucosal wave and phonatory function of the larynx.

Hylan B is promising as a soft tissue scaffold for vocal fold regeneration for the same reasons that have made it a candidate for a vocal fold augmentation material. Rabbit larynges injected with hylan B showed viscoelastic properties similar to those of natural rabbit vocal folds.<sup>10</sup> Rabbit vocal folds injected with hylan B showed persistence of hylan B at 1 year. Rabbit larynges injected with hylan B showed fibroblast infiltration and collagen deposition into the injected area.<sup>3</sup> It is possible that fibroblast expression of extracellular matrix in the layers of the vocal fold lamina propria varies with local environmental tissue pressure or velocity gradients. Infiltrating fibroblasts in submucosally placed hylan B gel might be exposed to an environment similar to native vocal fold, promoting differential extracellular matrix production that replicates the matrix in the layers of native lamina propria.

In the present study, the experimental animals had restoration of mucosal waves upon submucosal in-



Vocal folds at 12 weeks. **A)** Right (noninjected) vocal fold of experimental animal shows normal lamina propria. **B)** Left (hylan B–injected) vocal fold of experimental animal shows full regeneration of epithelium and lamina propria. Residual focus of hylan B is visible just superficial to muscular layer. **C)** Left (saline–injected) vocal fold of control animal shows epithelial regeneration with underlying dense scar tissue and no lamina propria regeneration.

jection of hylan B into the mucosalized neocord due to immediate restoration of tissue volume with material with viscoelastic properties similar to those of native lamina propria. The initial gross asymmetry of the waves in the experimental animals is probably due to volume and viscoelastic mismatches between the noninjected and injected folds. The volume mismatch occurs because the volume of hylan B injected only approximates the volume of the soft tissue deficit. The viscoelastic mismatch occurs because the ablated tissue was heterogeneous, including mucosa, lamina propria, and muscle, each with different viscoelastic properties. Replacing the ablated volume with hylan B, which has homogeneous viscoelastic properties, resulted in vocal folds that formed waves but showed gross asymmetry to the noninjected vocal folds. Over 12 weeks, the implanted hylan B was replaced with regenerated lamina propria and infiltrated fibroblasts that maintained tissue volume and allowed for function as measured by phonation at 12 weeks after injection. Histologic examination demonstrated only scattered foci of hylan B present at 12 weeks after injection, indicating that mucosal wave formation at that time was due to regenerated tissue. Mucosal waves in the experimen-

tal animals at 12 weeks after injection were nearly symmetric, suggesting that the regenerated vocal fold tissue observed histologically has viscoelastic properties closer to those of the noninjected vocal folds than to those of pure hylan B. The presence of a normal-sounding bark in the hylan B–injected animals is not a measure of the presence of mucosal waves; however, it does suggest that there was not substantial tissue volume loss during the 12 weeks between injection and phonation. The 1 experimental animal that required simultaneous superior and recurrent laryngeal nerve stimulation for formation of mucosal waves had a left vocal fold that was inferiorly displaced relative to the right fold. This finding correlates with the anteriorly subluxated left arytenoid cartilage and may be due to scar contracture in the wound bed after laser ablation. The fact that despite adequate medial regeneration of the left vocal fold in this animal a mucosal wave could not be obtained until it was elevated to the level of the contralateral fold by the addition of superior laryngeal nerve stimulation suggests that regeneration is more effective in the axial plane than in the vertical plane. Arytenoid cartilage repositioning is probably more effective than augmentation or regeneration for cor-

recting vertical position discrepancies between the vocal folds.

Although the descriptive nature of this study is a limiting factor, the results are promising. Since this work was performed, additional hyaluronic acid derivatives have been investigated for improved mechanical and degradative properties.<sup>11-14</sup> It will be exciting to test these in a canine model with an aim toward human use.

#### REFERENCES

1. Gray SD, Titze IR, Chan R, Hammond TH. Vocal fold proteoglycans and their influence on biomechanics. *Laryngoscope* 1999;109:845-54.
2. Hallén L, Dahlqvist Å, Laurent C. Dextranomers in hyaluronan (DiHA): a promising substance in treating vocal cord insufficiency. *Laryngoscope* 1998;108:393-7.
3. Hallén L, Johansson C, Laurent C. Cross-linked hyaluronan (hylan B gel): a new injectable remedy for treatment of vocal fold insufficiency — an animal study. *Acta Otolaryngol* 1999;119:107-11.
4. Catten M, Gray SD, Hammond TH, Zhou R, Hammond E. Analysis of cellular location and concentration in vocal fold lamina propria. *Otolaryngol Head Neck Surg* 1998;118:663-7.
5. Hammond TH, Zhou R, Hammond EH, Pawlak A, Gray SD. The intermediate layer: a morphologic study of the elastin and hyaluronic acid constituents of normal human vocal folds. *J Voice* 1997;11:59-66.
6. Webb K, Li W, Hitchcock RW, Smeal RM, Gray SD, Tresco PA. Comparison of human fibroblast ECM-related gene expression on elastic three-dimensional substrates relative to two-dimensional films of the same material. *Biomaterials* 2003;24:4681-90.
7. Berke GS, Moore DM, Hantke DR, Hanson DG, Gerratt BR, Burstein F. Laryngeal modeling: theoretical, in vitro, in

#### CONCLUSIONS

Submucosal injection of hylan B gel in the lasered canine vocal fold immediately restored tissue volume and mucosal waves. Over 12 weeks, hylan B was replaced by regenerated vocal fold tissue that retained the ability to form mucosal waves with phonation. Hylan B gel may have utility as a soft tissue scaffold for rehabilitation of phonatory function in vocal folds with lamina propria defects.

vivo. *Laryngoscope* 1987;97:871-81.

8. Atala A. Regenerative medicine and urology. *BJU Int* 2003;92(suppl 1):58-67.

9. Kanemaru SI, Nakamura T, Omori K, et al. Regeneration of the vocal fold using autologous mesenchymal stem cells. *Ann Otol Rhinol Laryngol* 2003;112:915-20.

10. Hertegård S, Dahlqvist Å, Laurent C, Borzacchiello A, Ambrosio L. Viscoelastic properties of rabbit vocal folds after augmentation. *Otolaryngol Head Neck Surg* 2003;128:401-6.

11. Shu XZ, Liu Y, Palumbo F, Prestwich GD. Disulfide-crosslinked hyaluronan-gelatin hydrogel films: a covalent mimic of the extracellular matrix for in vitro cell growth. *Biomaterials* 2003;24:3825-34.

12. Hansen JK, Thibeault SL, Walsh JF, Shu XZ, Prestwich GD. In vivo engineering of the vocal fold extracellular matrix with injectable hyaluronic acid hydrogels: early effects on tissue repair and biomechanics in a rabbit model. *Ann Otol Rhinol Laryngol* 2005;114:662-70.

13. Jia X, Yeo Y, Clifton RJ, et al. Hyaluronic acid-based microgels and microgel networks for vocal fold regeneration. *Biomacromolecules* 2006;7:3336-44.

14. Duflo S, Thibeault SL, Li W, Shu XZ, Prestwich GD. Vocal fold tissue repair in vivo using a synthetic extracellular matrix. *Tissue Eng* 2006;12:2171-80.