Functional testing of a tissue-engineered vocal fold cover replacement

Jennifer L. Long, Juergen Neubauer, Zhaoyan Zhang, Patricia Zuk, Gerald S. Berke and Dinesh K. Chhetri

Otolaryngology -- Head and Neck Surgery 2010 142: 438
DOI: 10.1016/j.otohns.2009.11.020

Otolaryngology–Head and Neck Surgery (2010) 142, 438-440

SHORT SCIENTIFIC COMMUNICATION

Functional testing of a tissue-engineered vocal fold cover replacement

Jennifer L. Long, MD, PhD, Juergen Neubauer, PhD, Zhaoyan Zhang, PhD, Patricia Zuk, PhD, Gerald S. Berke, MD, and **Dinesh K. Chhetri, MD,** Los Angeles, CA

No sponsorships or competing interests have been disclosed for this article.

ABSTRACT

OBJECTIVES: Tissue engineering may provide a treatment for severe vocal fold scars. This study quantifies mechanical properties and demonstrates vibration of a tissue-engineered vocal fold cover replacement.

METHODS: Tissue-engineered constructs were produced from fibrin and adipose-derived stem cells. Optimized bilayered constructs contained epithelial and mesenchymal cell phenotypes in a stratified geometry. For comparison, homogeneous constructs did not have epithelial differentiation. Elastic modulus was determined using indentation. Immunohistochemical labeling for type I collagen was performed. A bilayered construct was also tested in phonation in an excised larynx model.

RESULTS: Bilayered vocal fold cover replacements had indentation moduli similar to human vocal fold covers (mean construct modulus 6.8 kPa). Collagen deposition occurred in the middle of the construct. Homogeneous constructs had a mean modulus of 8.3 kPa, and collagen was concentrated at the surface. An excised larynx with unilateral vocal fold cover replacement phonated and exhibited mucosal waves at physiologic airflow.

CONCLUSION: Bilayered tissue-engineered constructs were produced that exhibited indentation modulus, microstructure, and vibration similar to that exhibited by human vocal fold covers.

Tissue engineering may provide treatment for vocal fold scarring. We propose that replacing the entire vocal fold cover may be more effective than addressing only the lamina propria in severe cases. We have produced a stratified tissue-engineered construct resembling the vocal fold epithelium and lamina propria using adipose-derived stem cells (ASC) in fibrin.[1] With epidermal growth factor (EGF) and an air interface, the ASC differentiate into epithelial cells near the surface and mesenchymal cells within the construct bulk. We now assess whether this candidate cover replacement has mechanical and vibratory properties similar to the native vocal fold.

Methods

Institutional Review

The UCLA Institutional Review Board approved the use of donated human lipoaspirate, cryoprecipitate, and cadaveric larynges.

Fibrin-ASC Constructs

ASC were isolated from lipoaspirate and cultured.[2] For fibrin constructs, cryoprecipitate was mixed with ASC and thrombin.[1] Three hundred μL was polymerized within Transwell inserts (Cole-Parmer, Vernon Hills, IL), then concentrated ASC were added to the surface. Half of the constructs were cultured with an air interface and were supplied 10 ng/mL EGF and 10% fetal bovine serum in the culture medium (bilayered group). The remaining constructs had EGF-free culture medium and were submerged under liquid (homogeneous group). All were harvested at two weeks.

For immunohistochemistry, samples were frozen, sectioned, and fixed onto slides. After blocking with goat serum, a rabbit antibody to type I collagen (DAKO, Denmark) was applied and detected with a goat-anti-rabbit fluorescein isothyocyanate–conjugated antibody.

For a phonating construct, a 3 × 1 cm rectangular well was scored in the base of a sterile culture dish. Cryoprecipitate-ASC-thrombin mixture was pipetted into the well. After the mixture gelled, additional concentrated ASC were added to the surface. Culture medium containing EGF bathed the gel on all sides but not on the surface. The sample was harvested at three weeks.

Indentation

A 1-mm indenter tip mounted onto a force transducer indented the construct surface in 0.025-mm steps until reach-

Received March 26, 2009; revised November 12, 2009; accepted November 13, 2009.

0194-5998/$36.00 © 2010 American Academy of Otolaryngology–Head and Neck Surgery Foundation. All rights reserved.
doi:10.1016/j.otohns.2009.11.020

ing 0.6 mm. Unloading proceeded in reverse, and five cycles were repeated for each sample. Elastic modulus E was calculated from the unloading slope of each force-indentation curve using the Hertzian model for a flat punch indenter:[3]

$$E = \frac{1 - v^2}{2R}\left(\frac{dF}{dh}\right)$$

where v is Poisson's compressibility ratio and R is indenter radius. Elastic modulus of the sample was taken as the mean from five cycles.

Excised Larynx Phonation

A cadaveric larynx was prepared by excising supraglottic structures. One vocal fold was dissected sharply to remove the anterior cover layer, including the medial surface. The rectangular tissue-engineered construct was sutured onto exposed vocalis muscle. The larynx was mounted on an air supply pipe. A high-speed camera recorded images at 2000 frames per second during phonation.

Results

Four cylindrical constructs in each group (8 total) were indented. Each measured approximately 6.5 mm in diameter and 7 mm thick. The mean elastic modulus of the bilayered group was 6.8 kPa (standard deviation 2.8 kPa), and the homogeneous group mean was 8.5 kPa (standard deviation 1.5 kPa). The groups did not differ significantly by Wilcoxon rank-sum test.

Immunohistochemistry demonstrated type I collagen deposition in both groups. The bilayered group had collagen labeling in the middle portion of the gel, away from the surface. The homogeneous group had stronger collagen labeling near the gel surface (Fig 1).

The tissue-engineered construct withstood handling, suturing, and shearing from airflow through the glottis. Audible phonation of the excised larynx with vocal fold cover replacement commenced at airflow of 1140 mL/second and subglottic pressure of 8 cm water. High-speed video of the phonating larynx showed vibrations of both the normal left vocal fold and the injured right vocal fold, with the construct attached. An inferior-to-superior mucosal wave was seen on both sides. Closure was incomplete. Mucosal wave of the right vocal fold cover appeared dampened in the midportion by a stitch that secured it to the deeper structures. Still images from a single vibratory cycle are shown in Figure 2.

Discussion

The defining function of the vocal fold cover is phonation. The excised larynx preparation is a useful screening tool to judge phonation of biomaterials or tissue engineered constructs for vocal fold replacement. We have found that our bilayered vocal fold cover construct vibrates at physiologic subglottic pressure and airflow.

Vocal fold oscillation derives from underlying structure and basic mechanical properties, most notably elastic modulus. We have measured elastic modulus of excised vocal folds with indentation, finding a mean elastic modulus of 4.8 kPa.[3] The elastic modulus of our epithelialized construct is similar, at 6.8 kPa. A closely matching modulus is requisite for phonatory vibration with physiologic subglottic pressure.[4] Other viscous properties are also critical for high-speed oscillation and remain to be explored using different methods.

The limited number of samples precludes drawing conclusions about mechanical differences between the homogeneous and bilayered samples, and the moduli were statis-

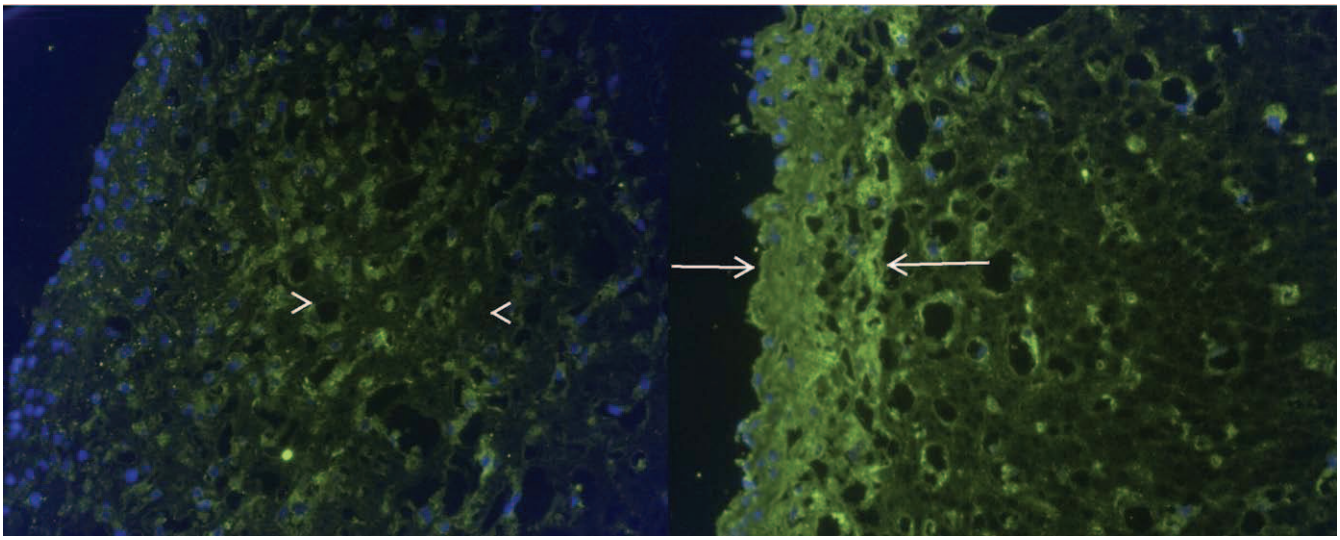

Figure 1 Immunohistochemical labeling for type I collagen in fibrin-ASC tissue constructs. Bilayered construct on the left shows green collagen labeling in the middle segment (*arrowheads*). Homogeneous construct on the right shows intense labeling near the surface (*arrows*). In both, nuclei are labeled blue. (Original magnification: ×20.)

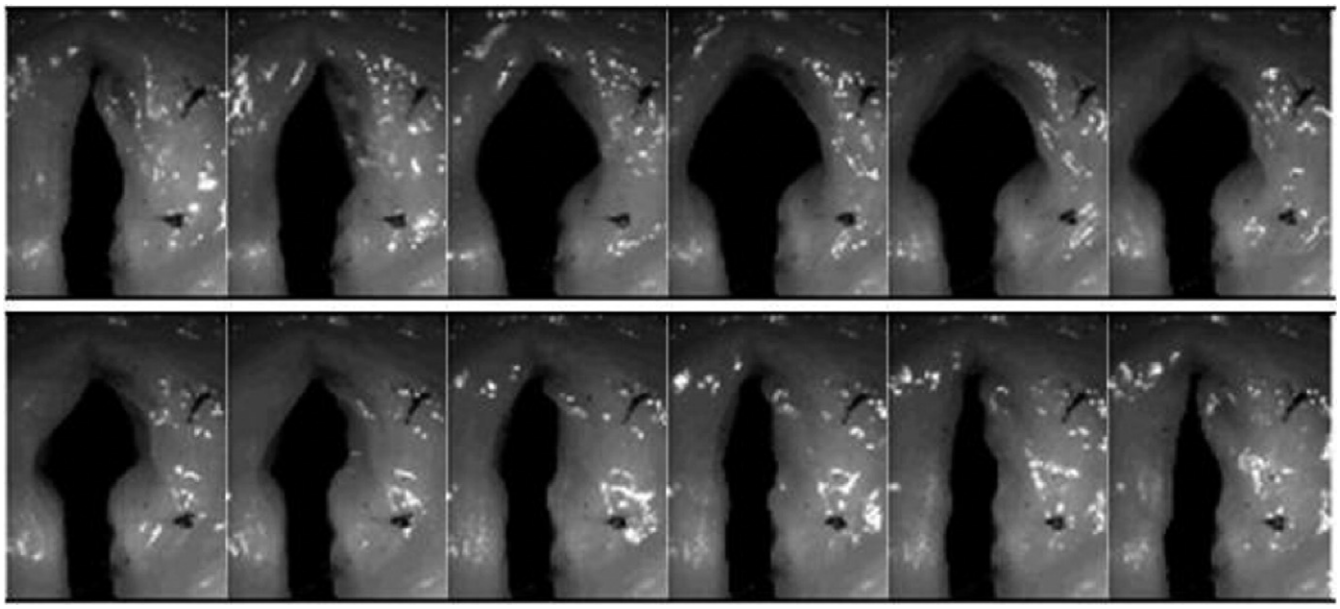

Figure 2 High-speed camera images of excised larynx phonation, with the right vocal fold cover replaced by a tissue-engineered construct. Anterior commissure is at the top of each image. Two stitches mark the construct corners in each image. Images are selected from a single glottic cycle. Modulation of the glottal opening is seen.

tically indistinguishable. Immunohistochemistry for type I collagen suggested greater surface collagen deposition in the homogeneous construct, which would be expected to increase elastic modulus on indentation. Collagen labeling in the bilayered construct occurred only in the deeper portion of the construct, resembling the vocal fold microstructure with the highest collagen density in the middle and deep lamina propria.

Conclusions

A tissue-engineered vocal fold cover was tested for functionally relevant properties in vitro. Elastic modulus and collagen microstructure were similar to human vocal fold covers. The construct oscillated and withstood physiologic shear airflow when attached to an excised larynx model of phonation. This construct may be suitable for vocal fold cover replacement.

Author Information

From the Division of Head and Neck Surgery (Drs. Long, Neubauer, Zhang, Berke, and Chhetri), and Division of Plastic Surgery (Dr. Zuk), David Geffen School of Medicine at UCLA, Los Angeles, CA.

Corresponding author: Jennifer Long, MD, PhD, Division of Head and Neck Surgery, David Geffen School of Medicine at UCLA, 650 Charles Young Dr., CHS room 62-132, Los Angeles, CA 90095.

E-mail: jlong@mednet.ucla.edu.

This article was presented at the 2009 AAO–HNSF Annual Meeting & OTO EXPO, San Diego, CA, October 4-7, 2009.

Author Contributions

Jennifer L. Long, conception, design, implementation, and manuscript preparation; **Juergen Neubauer,** design of methods, data acquisition, manuscript revision and approval; **Zhaoyan Zhang,** design of methods, data acquisition, manuscript revision and approval; **Patricia Zuk,** design of methods, manuscript revision and approval; **Gerald S. Berke,** study conception, manuscript revision and approval; **Dinesh K. Chhetri,** study conception, data analysis, manuscript revision and approval.

Disclosures

Competing interests: None.

Sponsorships: None.

References

1. Long JL, Zuk P, Berke GS, et al. Epithelial differentiation of adipose-derived stem cells for laryngeal tissue engineering. Laryngoscope 2010; 120:125–31.
2. Zuk PA, Zhu M, Mizuno H, et al. Multilineage cells from human adipose tissue: implications for cell-based therapies. Tissue Engineering 2001;7:211–26.
3. Chhetri DK, Zhang Z, Neubauer J, et al. Measurement of Young's modulus of vocal folds by indentation. J Voice 2009. In press.
4. Zhang Z. Characteristics of phonation onset in a two-layer vocal fold model. J Acoust Soc Am 2009;125:1091–102.