An In Situ, In Vivo Murine Model for the Study of Laryngotracheal Stenosis

Alexander T. Hillel, MD; Daryan Namba, BS; Dacheng Ding, MD, PhD; Vinciya Pandian, PhD; Jennifer H. Elisseeff, PhD; Maureen R. Horton, MD

IMPORTANCE Laryngotracheal stenosis (LTS) lacks an ideal animal model to study its unique wound-healing pathophysiology and the effect of interventions.

OBJECTIVE To present an in vivo, in situ mouse model of LTS that can be used to investigate its pathophysiology, mechanisms, and interventions for treatment.

DESIGN, SETTING, AND SUBJECTS Prospective controlled animal study performed at an academic animal research facility on 87 C57BL/6 mice.

INTERVENTIONS Experimental mice (n = 40) underwent bleomycin-coated wire-brush injury to the larynx and trachea, while mechanical injury controls (n = 32) underwent phosphate-buffered saline-coated wire-brush injury. Normal controls (n = 9) underwent no intervention, and mock surgery controls (n = 6) underwent anterior transcervical tracheal exposure only. Laryngotracheal complexes were harvested at days 7, 14, and 21 after injury. At the respective time points, mice in the chemomechanical and mechanical injury groups were killed, and their laryngotracheal complexes were harvested for histologic analysis. Normal and mock surgery controls were killed and then underwent histologic analysis.

MAIN OUTCOMES AND MEASURES The primary outcome measure was lamina propria thickness.

RESULTS The chemomechanical injury group maintained a significant increase in lamina propria thickness through day 21 compared with uninjured controls at day 7 (82.7 vs 41.8 μm; P < .05), day 14 (93.5 vs 26.0 μm; P < .05), and day 21 (91.2 vs 40.8 μm; P < .05). Compared with the mechanical injury group, the chemomechanical injury group demonstrated a significantly increased thickness at 21 days (91.2 vs 33.7 μm; P < .05).

CONCLUSIONS AND RELEVANCE Chemomechanical initiation of fibrosis in situ creates a viable mouse model of LTS that incorporates the physiologic circulatory supply and airflow. This small-animal model may be used to investigate the pathogenesis and inflammatory mechanisms of iatrogenic LTS and test therapeutic interventions to reverse or reduce the development of fibrosis.
Laryngotracheal stenosis (LTS) results in significant morbidity and can rapidly progress to life-threatening airway compromise. It has a number of causes, including iatrogenic or postintubation and/or posttracheotomy injury, autoimmune disease, irradiation, and idiopathy. Postintubation and/or posttracheotomy LTS is the most frequently reported type and is initiated by a combination of mucosal injury from and foreign-body reaction to the endotracheal or tracheostomy tube. The initial injury triggers wound healing with inflammation, proliferative, and maturation phases. In LTS, these stages of physiologic wound healing become pathogenic, leading to considerable tissue remodeling and the formation of permanent scar tissue in the airway.

Laryngotracheal stenosis lacks an ideal animal model to study its unique wound-healing pathophysiology and the effect of interventions. While larger-animal models have been commonly studied, mouse models generally allow for greater numbers, lower costs, and genetic and molecular investigation. Ghosh et al and Richter et al have recently used an LTS mouse model, developed by Richter et al., to suggest an immune cell-mediated process and to demonstrate the effect of steroid treatment at reducing LTS. However, the ex situ model lacks elements that provide important contributions to the development of stenosis, namely laryngotracheal vascularity, an intact pulmonary tree, and anatomic proximity to gastrointestinal tract.

To address limitations in current ex situ mouse models, we developed an in situ, in vivo LTS model using a tracheal cut-down to visualize a bleomycin-coated wire-brush application to mechanically and chemically injure the tracheal mucosa. Bleomycin is a cytoxic chemotherapeutic agent with an adverse-effect profile that includes pulmonary fibrosis. Although bleomycin falls under the same “antitumor antibiotic” classification as mitomycin C, with its antifibroblast proliferation effect, bleomycin at high doses can cause pulmonary fibrosis and dural fibrosis. Since it was first noted to cause pulmonary fibrosis in initial clinical trials, application of bleomycin has been used in experimental models to study the mechanism of fibrosis, including in the lungs and skin. Bleomycin-induced dermal thickening is a similar process to lamina propria (LP) thickening in LTS, suggesting a potential role for bleomycin in a mouse model of LTS. An in situ LTS model is ideal to study tracheal fibrosis because airflow, the physiological circulatory system, and gastroesophageal reflux elements remain intact. We hypothesize that use of the in situ LTS mouse model will provide insight into the development of, mechanisms behind, and ultimately interventions for the treatment of LTS.

Methods

Experimental Design

This study was approved by the Johns Hopkins University Animal Care and Use Committee (MO12M354). Eighty-seven C57BL/6 mice (Charles River Laboratory) were used in this study. The experimental group (n = 40) underwent bleomycin-coated wire-brush injury to the larynx and trachea, while control group 1 (n = 32) underwent phosphate-buffered saline (PBS)-coated wire-brush injury. Control group 2 (n = 9) underwent no intervention and represented normal controls at evaluation each time point. A mock surgery control group (n = 6) was included to isolate the impact of the tracheal cut-down on potential transmural injury of the tracheal LP at days 7 and 14. The primary outcome measure was LP thickness. Mice were killed, and laryngotracheal complexes were harvested at days 7, 14, and 21 after injury in all groups for histologic analysis (n = 3 for each group at each time point). Laryngotracheal complexes were also harvested at days 1, 4, 7, 14, and 21 days after injury in the experimental group and control group 1 for gene expression analysis (n = 3 for each group at each time point). All specimens were analyzed in a blinded fashion. There were 16 deaths in the experimental group, 8 in control group 1, and 0 in control group 2 that occurred outside of planned euthanasia time points.

Surgical Technique

Mice underwent tracheal cut-down for direct visualization of the trachea during intubation with a 22-gauge angiocatheter (Smiths Medical). Next a 0.02-inch-diameter steel wire brush (Mill-Rose Co) coated in bleomycin, 1U/mL (APP Pharmaceuticals) was placed through the angiocatheter into the trachea. The angiocatheter was then retracted into the oropharynx and the laryngotracheal mucosa was scraped directly with the wire brush. The steps to the surgical technique are depicted in Figure 1. The brush was placed in the bleomycin solution 5 times and a total of 15 passes along the laryngotracheal mucosa was made with the brush for each mouse. Control group 1 underwent 15 passes with a wire brush coated in PBS.

Histologic and Statistical Analysis

Laryngotracheal complexes were fixed in 10% formalin, and then each specimen was embedded in paraffin. Slides were made from 5-μm-thick sections cut through in an axial plane, which were then stained with hematoxylin–eosin. Specimens were assessed to measure LP thickness and identify fibroblasts, collagen production, angiogenesis, and inflammatory cell infiltrate. To determine LP thickness, the subglottis was split into 5 equal segments (Figure 2), and the thickest portion of each segment was recorded. Measurements were performed from the medial aspect of the tracheal cartilage to the basement membrane of the epithelium at ×40 magnification. Means and standard deviations were calculated. Statistical significance between groups was determined by the Mann-Whitney-Wilcoxon test and defined as P < .05.

Results

Sustained LTS From Chemomechanical Injury

In the chemomechanical LTS group, mice demonstrated a thickened subepithelial LP with an initial increase in inflammatory infiltrate including lymphocytes, neutrophils, and macrophages at day 7 (Figure 3). Inflammatory cells peaked at day 7 and then transitioned to increased collagen deposition with a decreased cellular pattern (Figure 3). The initial inflam-
Tracheal cut-down is performed to visualize the trachea. B, Next, the mouse is intubated with an angiocatheter, the tip visualized within the trachea. C, Finally, the wire brush is passed through the angiocatheter, which is retracted slightly to allow for brush injury along the larynx and trachea.

A, Hematoxylin-eosin–stained representative section (original magnification ×10) of subglottic stenosis divided into 5 equal arc segments (I-IV). B-F, Thickness measurements of the lamina propria (black spanning bars) and epithelium (green spanning bars) were taken at the thickest locations in each arc segment (original magnification ×40). Lamina propria and epithelium means were calculated for each mouse at each time point after injury (days 7, 14, and 21), and the average thicknesses of 3 mice were compared with the 2 control groups at each time point.
intact epithelium with a normal LP ranging between 1 and 4 cell layers thick. The epithelial thickness in normal controls was significantly less than that in the PBS controls (19.1 vs 31.3 μm; \( P < .05 \)), and experimental group (19.1 vs 31.9 μm; \( P < .05 \)). There was no significant difference in epithelial thickness at day 14 or day 21.

**No Appreciable LP Injury From Mock Surgery**
The mock surgery controls were assessed at days 7 and 14 and compared with the chemomechanical injury group (Figure 4). There was no appreciable injury to the LP or epithelium in mock surgery controls at either time point; the mock surgery sections (Figure 4A and B) closely resembled those of the normal controls (Figure 3A).

**Measurable Increase in LP Thickness Following Chemomechanical Injury**
The mechanical injury group did not demonstrate any difference in LP thickness at days 7, 14, and 21 compared with uninjured controls. The chemomechanical injury cohort maintained a significant increase in thickness at days 7 (82.7 vs 41.8 μm; \( P < .05 \)), 14 (93.5 vs 26.0 μm; \( P < .05 \)), and 21 (92.2 vs 40.8 μm; \( P < .05 \)), compared with uninjured mice. When compared with the mechanical injury group, the chemomechanical injury group had a similar LP thickness at day 14 (93.5 vs 65.7 μm) and then demonstrated a significantly increased thickness at day 21 (91.2 vs 33.7 μm; \( P < .05 \)). Figure 5 graphically shows the difference in LP thickness between the mice with chemomechanical injury, mechanical injury, and uninjured controls at days 7, 14, and 21.

**Discussion**
While some studies suggest that the incidence of LTS after prolonged intubation is decreasing,12 2 recent prospective studies demonstrated an 11% incidence of subglottic stenosis in children and an 11% incidence of laryngotracheal injury in adults.13,14 Laryngotracheal stenosis can have a negative impact on quality of life; it presents an increased risk of tracheostomy; and treating it often requires multiple surgical procedures with associated morbidity.1,15 Surgical therapies, including serial dilation, cricotracheal resection, laryngotracheal reconstruction, and tracheostomy, have changed little
over the last 50 years, and there is inconsistent evidence of efficacy from medical therapies.\textsuperscript{7,16–18} The primary barrier in developing better therapies for LTS is that there still exists a very limited understanding of the mechanisms behind the critical fibrosis. While fibroblasts represent the end effector cell in LTS, by the time increased collagen deposition results in clinical stenosis, it is too late to effectively intervene medically.

Animal models of LTS can help elucidate its mechanisms and pathophysiology. Large-animal models of acquired LTS including rabbit, pig, and canine have been published demonstrating inflammatory markers and suggesting treatment effect.\textsuperscript{19–22} However, these large-animal models have limitations including variable response to injury and expensive animal costs.\textsuperscript{23–25} Recently, Richter et al\textsuperscript{8} developed an ex situ mouse model using heterotopic transplantation of tracheas with cauterized mucosa to initiate subglottic stenosis. This model demonstrated a significant thickening in the subepithelial connective tissue, or LP, with fibroblasts, angiogenesis, and a predominate lymphocytic infiltrate.\textsuperscript{8} The heterotopic transplant model has also been used in multiple studies by Ghosh et al\textsuperscript{6,7} that showed evidence of lymphocytes mediating the formation of tracheal granulation tissue and the successful use of steroid therapy to inhibit the granulation tissue.

The current in situ, in vivo mouse model expands on the ex situ model of Richter et al.\textsuperscript{8} The in situ model includes the natural circulatory route of inflammatory cells to the larynx and trachea and incorporates the effect of airflow biomechanical forces on epithelial and submucosal healing. We favored the use of a wire brush to cause laryngotracheal injury, as described by Ghosh et al,\textsuperscript{6} because of circumferential thickening and difficulty of using electrocautery in situ while optimizing mouse survival. As the model was being developed, initial mechanical injury alone was inconsistent in causing inflammation and furthering the development of stenosis over time. It was not significantly different from normal mouse tracheal LP thickness, so the chemotherapeutic agent, bleomycin, was added to accelerate the development of fibrosis. Similarly, chemical injury alone with intratracheal aerosolization of bleomycin resulted in normal appearing tracheobronchial mucosa, further supporting the need for chemomechanical injury. The chemomechanical injury cohort showed increased stenosis over time, typical of that seen in other models and in human experience.\textsuperscript{8,22,23} The initial increased inflammatory infiltrate in the chemomechanically injured mice transitioned to significantly greater collagen deposition and fibrosis over time compared with the mechanically treated mice, mock surgery mice, and untreated controls (Figures 3–5). Compared with untreated controls, the chemomechanical injury group maintained significantly greater LP thickness at 21 days (91.2 vs 40.8 μm; \textit{P} < .05) (Figure 3).

In summary, the chemomechanical initiation of LTS is an in situ, in vivo mouse model that may be used to investigate iatrogenic LTS. Similar to other mouse models, the in situ development of LTS can allow for rapid, relatively inexpensive, large experimental group studies with the potential to generate significant findings that translate to improved therapy for human LTS. Future studies using this in situ, in vivo model will aim to define the clinical effects of mouse LTS including mortality rate, respiratory rate, and oxygen saturation. Analysis of specific immune cells and cytokines in the acute inflammatory period could help to elucidate the sequence between injured epithelial cells and resultant fibroplasia. Ultimately, we plan to compare LTS in genetically altered mice to identify the mechanisms behind the inflammation and potentially immunomodulators that would prevent fibrosis from developing following injury (or dilation).

While this is the first study to our knowledge to successfully study the in situ development of LTS in a mouse, there are shortcomings to this model. It is a mechanical model requiring a surgical technique to initiate LTS, which makes it less consistent than a genetic or chemical model of disease. Furthermore, while the effects of bleomycin may not necessarily recapitulate the exact steps that occur in traumatic LTS, bleomycin is a well-accepted accelerator that is used in other respiratory models of fibrosis.\textsuperscript{10,24} One other drawback of this method is the 40% mortality rate of mice undergoing the experimental procedure. This mortality rate is in line with mouse models of pulmonary fibrosis induced by high-dose bleomycin.\textsuperscript{24} A moderately high mortality rate may become a strength of this model in future studies, with mortality rate reduction representing a key study outcome. Should a genetically altered or immunomodulator-treated group show improved survival, the reduction in mortality would signify a successful intervention.

**Conclusions**

Chemomechanical initiation of fibrosis in situ creates a viable mouse model of iatrogenic LTS that incorporates the...
effects of a physiologic circulatory supply, airflow, and gastrointestinal tract. This small-animal model may be used to further investigate the inflammatory steps preceding fibrosis. Defining the immunologic cascade leading to LTS would be a significant development in understanding normal and abnormal wound healing in the larynx and trachea. Further study with the in situ model should help clarify mechanisms behind LTS and identify therapies to reduce or reverse the development of fibrosis in the larynx and trachea.