A Novel Murine Model for the Examination of Experimental Subglottic Stenosis

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Objective: To develop a novel murine model of acquired subglottic stenosis (SGS) using heterotopic transplanted laryngotracheal complexes (LTCs).

Design: Pilot randomized controlled animal study.

Subjects: Forty-eight C57BL/6 mice.

Interventions: Twenty-four donor C57BL/6 mice underwent LTC harvesting. The LTCs were then transplanted deep to a cutaneous dorsal flap in 24 allogenic recipients. Sixteen LTCs underwent direct subglottic injury before transplantation, while 8 control LTCs were transplanted without injury. Transplanted LTCs were harvested 1, 2, 3, and 4 weeks after surgery. Tissues were fixed and cut transversely in 6-μm sections from the larynx to the second tracheal ring. Movat pentachrome staining showed random distributions and high concentrations of connective tissue within the lamina propria of the subglottis. Vascular endothelial growth factor 164 (VEGF 164) and transforming growth factor β1 (TGF-β1) immunohistochemistry was performed on representative sections.

Results: Lamina propria thickness was significantly greater in transplanted LTCs 3 and 4 weeks after injury compared with controls (P = .03, P = .01, respectively). Combined results (groups harvested at 1-4 weeks) revealed a significant difference between all 8 control animals and all 16 experimental animals (P < .001). Epithelial thickness was also greater in the transplanted LTCs 2, 3, and 4 weeks after injury to the subglottis compared with controls (P = .04 for weeks 2 through 4). Movat pentachrome staining showed random distributions and high concentrations of connective tissue within the lamina propria of the subglottis. The VEGF 164 and TGF-β1 staining patterns were consistent with previous in vivo models of SGS.

Conclusion: Heterotopic transplanted LTCs in mice can provide an inexpensive and flexible model for experimental investigation of acquired SGS.


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ANIMAL MODELS FOR THE INVESTIGATION OF ACQUIRED SUBGLOTTIC STENOSIS (SGS) ARE UNIFORMALLY AFFlicted BY TECHNICAL CONSTRAINTS AND BY HIGH MORTALITY RATES.1–3 PORCINE, RABBIT, FERRET, AND CANINE SPECIES HAVE BEEN USED IN THE CREATION AND EXAMINATION OF SGS FOLLOWING DIRECT IN SITU MUCOSAL INJURY.4–8 FURTHERMORE, THESE ANIMAL MODELS HAVE BEEN USED TO EXPLORE VARIOUS OPEN AND ENDOSCOPIC SURGICAL PROCEDURES TO TREAT SGS. HOWEVER, THESE ANIMAL MODELS ARE ACCOMPANIED BY KNOWN RISKS OF SURGICAL VARIATION AND ERROR.9–11 THEREFORE, DESPITE THE CURRENT USE OF SEVERAL SPECIES AND TECHNIQUES, THE IDEAL MODEL FOR THE INVESTIGATION OF SGS REMAINS TO BE DESCRIBED.

HISTORICALLY, THE MOUSE MODEL HAS BEEN THE WORKHORSE FOR EXPERIMENTAL STUDY. IN PARTICULAR, DEVELOPMENTAL RESEARCH ON THE TRACHEA, BRONCHI, AND LUNGS HAS USED THE MURINE MODEL.12,13 THIS MAY PARTIALLY BE ATTRIBUTED TO EASE IN HANDLING, REDUCED EXPENSE, RAPID DEVELOPMENT, AND IMPROVED BREEDING CAPACITY OF THIS SPECIES COMPARED WITH LARGER ANIMALS.12,13 UNFORTUNATELY, LIMITATIONS IN ANIMAL SIZE AND AIRWAY CALIBER PREVENT THE USE OF THIS SPECIES FOR IN SITU EXAMINATION OF SUBGLOTTIC DISEASE. SOME RESEARCHERS HAVE PERSISTED IN EXPLOITING THE VIRTUES OF THE MOUSE MODEL BY USING AN EX SITU APPROACH TO STUDY BIOLOGIC FUNCTION OF THE TRACHEA.14,15 TO OUR KNOWLEDGE, THIS EX SITU APPROACH HAS YET TO BE USED IN THE EXAMINATION OF SGS. IN THIS STUDY, WE AIMED TO DEVELOP A FUNCTIONAL MODEL OF SGS USING HETEROtOPOIC TRANSPLANTED MOUSE LARYNGOTRACHEAL COMPLEXES (LTCs). WE HYPOTHESIZED THAT THIS NOVEL TECHNIQUE MAY PROVIDE AN EFFICIENT TOOL FOR INVESTIGATING THE DEVELOPMENT AND TREATMENT OF SGS.

METHODS

MOUSE MODEL

This study was approved by the Animal Care and Use Committee at Cincinnati Children's Hospital. A pilot randomized controlled animal study was undertaken. Forty-eight C57BL/6 mice were used in the creation and examination of acquired SGS.
Hospital Medical Center. All animals were housed in an Association for Assessment of Laboratory Animal Care–approved facility with water and regular food ad libitum. Forty-eight C57BL/6 mice (Taconic Farms, Inc, Hudson, New York) were used in the study. Twenty-four “donor” C57BL/6 mice underwent dissection and harvesting of their airways as a single unit. The airways were then transplanted into a subcutaneous pocket on the dorsum of an additional 24 genetically equivalent “recipient” mice. Before transplantation, 16 animals underwent direct subglottic injury, while 8 control LTCs were transplanted without injury. The transplanted airways were harvested 1, 2, 3, and 4 weeks after surgery. Two tracheas were removed from recipient animals for use as nontransplanted control specimens (Table).

### SURGICAL TECHNIQUE

Following isoflurane euthanasia, a vertical midline incision was created from the mentum to the sternum in each donor mouse. Under microscopic guidance, the thyroid, cricoid, and high tracheal cartilages (LTCs) were exposed with meticulous dissection of the overlying strap muscles, thyroid gland, and thymus (Figure 1A and B). The entire thyroid cartilage, cricoid cartilage, and at least the first 3 tracheal rings were subsequently removed from the adjacent suprahoid attachments, esophagus, and distal trachea.

The experimental group was created by direct injury to the subglottis before removal of the LTCs. This was performed under microscopic guidance using a monopolar electrocautery with a fine-needle tip aimed through a high vertical tracheotomy (2-3 tracheal rings). Care was taken not to open the anterior cricoid plate. The needle tip was placed directly at the right posterior cricoid lamina mucosa and was ignited for less than 1 second at a setting of 1 to 3 W. The injury created with 3 W was performed on the first 2 experimental animals and was determined to be equivalent (by microscopic surgical view) to the injury (eschar) created with 1 W. A setting of 1 W was subsequently used on the remaining subjects. Eight animals served as controls with direct harvesting and transplanting of the LTCs without injury. Recipient mice were anesthetized with isoflurane and surgically prepared along their dorsum. A small horizontal incision was made at the scruff of the neck between the opposing scapula (arrow). The donor trachea was centered in the pocket, and the outer layer of skin was closed with tissue glue (Nexband; Veterinary Products Laboratories, Phoenix, Arizona) (Figure 1C).

### LTC HARVESTING AND PROCESSING

Transplanted LTCs were harvested 1, 2, 3, and 4 weeks after surgery by random selection of 4 experimental mice and 2 controls for each harvesting period. Recipient C57BL/6 mice were euthanized by carbon dioxide inhalation. The transplanted LTCs were harvested via the previously placed horizontal incision along the dorsum neck skin of the recipient mice (Figure 2).
and were fixed in 4% paraformaldehyde (Electron Microscopy Sciences, Hatfield, Pennsylvania) and phosphate-buffered saline solution for 18 to 24 hours at 4°C. Specimens were then dehydrated through a series of alcohol washes and embedded in vertical orientation into paraffin blocks. Using a microtome, 6-μm axial sections were cut from the level of the larynx to the second tracheal ring. Microscopic sections were stained with hematoxylin-eosin for microscopic analysis. Movat pentachrome stain (Poly Scientific R&D Corp, Bay Shore, New York) was used for staining connective tissue.

MOVAT PENTACHROME STAINING

The pentachrome method is used to stain connective tissues, including cartilage, elastic fibers, collagen, reticulum fibers, and muscle. A Movat pentachrome staining kit was used (Poly Scientific R&D Corp). Six-micrometer sections were deparaffinized, hydrated, placed in 1% alcian blue-acetic acid for 20 minutes to stain mucopolysaccharides or glycosaminoglycans; washed in running water; and then placed in alkaline alcohol for 2 hours to convert the alcian blue into monastral fast blue. These slides were then rinsed in 70% alcohol and placed in crocein-fuchsin working solution for 16 hours, which binds and stains elastic fibers and renders them purple to black. Following another rinse the next day, slides were placed in Weigert hematoxylin working solution for 15 minutes and in wood stain scarlet-acid fuchsin solution for nuclear staining. Slides were then rinsed in 0.5% acetic acid and were differentiated in 3% aqueous phosphotungstic acid for 10 to 20 minutes until collagen turned pale pink and the background substance again turned blue green. Following another rinse with 0.5% acetic acid and absolute alcohol, specimens were placed in Safran du Gatinais solution for 15 minutes, which binds and renders collagen yellow. All slides were then dehydrated and cleared with xylene. The end result of pentachrome staining is selective coloring of the extracellular components as shown in Figure 3.

MORPHOMETRIC AND STATISTICAL ANALYSES

At 20 magnification, representative digital images of the subglottic lumen were obtained for each harvested LTC following pentachrome staining. The subglottis was identified microscopically by locating the cricoid cartilage, a contiguous cartilaginous ring that could be distinguished from the C-shaped tracheal cartilage. Each experimental group, consisting of 4 LTCs, was harvested 1, 2, 3, or 4 weeks after transplantation. The LTCs of 2 control animals were also examined at each posttransplantation time. Images were obtained (DM4000B microscope; Leica Microsystems, Deerfield, Illinois; and SPOT Model RTKE Slide camera and SPOT software; Diagnostic Instruments, Inc, Sterling Heights, Michigan) and imported using commercially available software (MetaMorph Imaging Software version 6.2; Universal Imaging Corp, Westchester, Pennsylvania). Under 10 magnification, each section underwent microscopic measurement of the subglottis lamina propria and epithelial thicknesses by an investigator blinded to the group identification. Thickness of the lamina propria was measured at 5 equidistant sites along the subglottis. Lamina propria was defined as the area between the basement membrane and the perichondrium. The thickness of the epithelium, defined as the distance between the basement membrane and the luminal surface of the epithelium, was measured at 5 sites along the subglottis. Values for each LTC within a posttransplantation time group (2 control and 4 experimental) were combined to obtain the mean lamina propria and epithelial thicknesses for that group. Data were graphed as mean (SE), and statistical analysis was performed using the t test and assuming unequal variance to determine the statistical significance (P = .05) between each experimental and control group. A final evaluation of control vs experimental groups, combining all posttransplantation time groups, was performed using the same statistical methods.

IMMUNOHISTOCHEMISTRY

Microscopic sections of the subglottis from representative control and experimental animals were subjected to immunostaining using antibodies to transforming growth factor β1 (TGF-β1) and vascular endothelial growth factor 164 (VEGF 164). Experimental and control LTCs from each posttransplantation time group were examined.

TRANSFORMING GROWTH FACTOR β1

First, antigen retrieval was performed by treatment of tissue with 5% hyaluronidase (H3506 type I; Sigma-Aldrich Inc, St Louis, Missouri) for 1 hour. After rinses with triphosphate-buffered saline (TBS), the slide was incubated overnight at room temperature in primary antibody rabbit antiporcine TGF-β1
(T8250-13A; U.S. Biological, Swampscott, Massachusetts) at 1:200 dilution with TBS, 3% bovine serum albumin (BSA), and 1% normal goat serum (Vector Laboratories, Burlingame, California). Slides were rinsed in 0.2% TBS and Tween 20, and then incubated for 20 minutes in TBS, 3% BSA, and 0.9% sodium chloride. Endogenous peroxidase was blocked by incubating tissues in 1 mL of TBS and 3% BSA, 500 µL of hydrogen peroxide, and 10 mg of sodium azide for 20 minutes. Following another TBS rinse, tissues were incubated with a 1:200 dilution of the secondary antibody, biotinylated antirabbit IgG (Vector Laboratories), for 30 minutes. Slides were rinsed in TBS. A staining kit (ABC Vectastain kit, Vector Laboratories) with nickel and diaminobenzidine was used to reveal the expression of TGF-β1. Tissues were counterstained with nuclear fast red.

Results

Twenty-four recipient genetically equivalent mice were used in the study. All animals survived LTC transplantation and were euthanized immediately before harvesting the LTCs. At harvest, the LTCs were noted to be encapsulated in a highly vascular tissue matrix, without signs of infection. All LTC specimens appeared to contain intact cartilage, mucosa, and submucosal components on histologic evaluation. Pentachrome staining suggested increased levels of ground substance and collagen within the lamina propria of the subglottis of the experimental groups. This was particularly apparent in the experimental LTCs harvested 3 weeks after transplantation (Figure 3).

Figure 4 illustrates the mean lamina propria thicknesses of control and experimental animals at each post-transplantation harvest. There is an observable thick-
ness increase in each experimental group compared with controls. A significant difference was identified 3 weeks ($P = .03$) and 4 weeks ($P = .01$) after injury compared with controls. The 4 posttransplantation harvest groups were combined and compared with control LTCs, again demonstrating a statistically significant difference between lamina propria thicknesses in experimental vs control LTCs ($P < .001$).

Immunohistochemical staining was performed for TGF-$\beta_1$, a marker of acute inflammation and procollagen formation, and for VEGF 164, an angiogenic and wound healing promoter. A single animal from each control and experimental group was used to examine the expression of these cytokines in the subglottis. Reliable differences in the expression of VEGF 164 or TGF-$\beta_1$ could not be determined between control and experimental groups because of the insufficient number of animals and the variability in measuring signal density. However, these stains confirmed the presence of VEGF 164 in the submucosa of the subglottic lumen of treated LTCs and in the fibrous muscular attachments surrounding treated and untreated LTCs. The presence of VEGF 164 in these locations is consistent with the ongoing processes of wound healing and increased vasculogenesis at these sites after transplantation (Figure 6). Although not proven by these

Figure 4. The mean lamina propria thicknesses in control and experimental animals at each posttransplantation harvest. A, According to posttransplantation week. There was a significant difference between injured (experimental) and uninjured (control) subglottic lamina propria thicknesses, also a measure of stenosis in vivo animal models, in laryngotracheal complexes (LTCs) 3 weeks ($*P = .03$) and 4 weeks ($†P = .01$) after transplantation. B, Accumulated data of all control and experimental transplanted LTC lamina propria thicknesses, illustrating the significant difference between these 2 groups ($‡P < .001$).

Figure 5. The mean epithelial thicknesses of the subglottis in control and experimental animals at each posttransplantation harvest. A, According to posttransplantation week. There was a significant difference between injured (experimental) and uninjured (control) subglottic epithelial thicknesses in laryngotracheal complexes (LTCs) 2 weeks ($*P = .04$), 3 weeks ($†P = .03$), and 4 weeks ($‡P = .04$) after transplantation. B, Accumulated data of all control and experimental transplanted LTC epithelial thicknesses, illustrating the significant difference between these 2 groups ($§P < .001$).
studies, VEGF 164 is likely being expressed by endothelial cells of the newly forming vasculature. The TGF-β1 staining was predominantly located within chondrocytes and perichondrium of the cricoid cartilage of control and experimental LTCs in each posttransplantation time group.

**COMMENT**

The incidence of SGS among infants has waned during the past few decades, with some authors suggesting a rate of less than 0.6% annually. Still, acquired SGS remains one of the most common causes of stridor and upper airway obstruction in infants. Improved survival of premature infants may contribute to a future rise in the incidence of SGS, with endotracheal intubation and prolonged ventilation required to maintain life in these children. Although surgical management of SGS has achieved high success rates, this disease continues to present notable therapeutic challenges. The complexity of mucosal wound healing and the process of subglottic fibrosis has intrigued many investigators.

Subglottic stenosis is thought to arise from injury to the subglottic mucosa, submucosa, and cartilage from infection, direct trauma, perichondritis, mucosal ulceration, excessive endotracheal tube pressure, or repetitive mechanical stress created by prolonged intubation. The concept of an iatrogenic origin of SGS has been demonstrated in several animal models in which even minor injuries to the subglottis lead to profound stenosis over time. However, the cause of acquired SGS and the mechanisms of mucosal wound healing remain elusive. Human studies are impossible to construct because of many factors, including the low incidence of acquired SGS. Therefore, investigators have resorted to animal models of SGS. In numerous species, controlled injury to the subglottic mucosa leads to epithelial destruction and subglottic stenosis. In the context of these studies, it is important to consider the role of intrinsic factors that may predispose certain individuals to develop SGS.

**Figure 6.** Transforming growth factor β1 (TGF-β1) and vascular endothelial growth factor 164 (VEGF 164) immunohistochemistry of subglottic lumens of control and experimental laryngotracheal complexes (LTCs) after transplantation. Black stain indicates the location of signal intensity for both molecules; TGF-β1 is found predominantly within the perichondrium and cricoid cartilage, and VEGF 164 is expressed within the lamina propria and around the entire LTC transplant, consistent with ongoing processes of neovascularization and inflammation in these areas.
glottis has revealed pathologic patterns of acquired stenosis similar to that seen in the newborn human.\textsuperscript{2,3,5,7} Immunohistochemistry has confirmed common inflammatory pathways.\textsuperscript{19,20} Large animal models have been generated to translate experimental findings into human treatment algorithms.\textsuperscript{7} Although these studies have furthered our understanding of the development of acquired SGS, these models have limitations. Limitations of these animal models include high mortality rates from airway obstruction or anesthetic overdose, expensive animal handling fees, and variability among subjects.\textsuperscript{2,3,8} Mortality rates of 23\% to 37\% have been reported following the operative creation of SGS in rabbits.\textsuperscript{2,7} Airway caliber, the extent of luminal injury, and animal factors have been blamed.

In this study, we aimed to develop a model of SGS that was flexible, reliable, and inexpensive. The murine model has been used for numerous investigations of human disease to translate experimental findings to cures before testing in human subjects. New concepts pertaining to pulmonary development and pathologic conditions and to gastrointestinal mucosal healing have been derived from the mouse model.\textsuperscript{12,21} The wealth of biologic and molecular reagents for mouse models led us to develop a mouse model of SGS. Technical restraints limit in situ creation of injured mouse airways. Larger species (ferret, canine, chimpanzee, rabbit, and rat) experience high mortality rates from airway compromise, which would be expected to be exacerbated in an anatomically smaller model. The concept of creating a heterotopic transplantation model of SGS was devised to circumvent this limitation. Reductions in handling space and cost associated with the mouse model have been a serendipitous benefit.

Previous cases of experimental SGS have been developed by creating direct injury to an area or circumfer- ence of the subglottis using various techniques and tools. These investigations make apparent the need for a direct penetrating injury that disrupts the lamina propria and perichondrium to create a reliable model of acquired SGS.\textsuperscript{7} In the present model, we created intense focal injury to the posterior cricoid using monopolar cautery before transplanting the entire LTC into a genetically equivalent recipient. At harvest, the LTC was encapsulated in a stroma of highly vascular fibrotic tissue, allowing survival of the LTC even at the 4-week posttransplantation harvest. This finding was consistent with the high expression of VEGF 164 at the outer perimeter of the harvested LTC. The viability of the LTC was confirmed on histologic evaluation, demonstrating conservation of all LTC subcomponents. This included maintenance of the integrity of the trilaminar structure of the subglottic epithelium, lamina propria, and cartilage. Because of evidence of transplanted LTC viability, subglottic lumen measurements of treated (injured) and control LTCs could be obtained with confidence.

Several measures have been used to detect changes in subglottic lumen size in animal models of SGS. As a measure, the lamina propria thickness has been used, a location where fibroblast proliferation is most active in response to injury.\textsuperscript{10} Increased concentrations of collagen, glycosaminoglycans, and other connective tissue components have been identified in the lamina propria after injury.\textsuperscript{7} Elevated levels of TGF-\(\beta\) have been demonstrated in the lamina propria following subglottic injury. Transforming growth factor \(\beta\) has an effect on procollagen formation, fibroblast conversion, and extracellular matrix deposition.\textsuperscript{7} In the present study, Movat pentachrome staining demonstrated increased levels of various extracellular matrix components in the lamina propria. Although reliable measures of these components could not be obtained in this study, these observations suggested a similar pattern of subglottic changes in this heterotopic model.\textsuperscript{7} In future studies, more quantitative measures will be used to measure TGF-\(\beta\) expression to understand its role in SGS.

As in other models of SGS, this study demonstrated greater thickness of the lamina propria in the subglottic lumen of experimentally injured and explanted LTCs.\textsuperscript{10} Compared with controls, this was significantly different 3 and 4 weeks after transplantation and was consistent with human experience and in vivo animal studies\textsuperscript{22} illustrating greater stenosis with increased time. The epithelium was also thicker at each posttransplantation time in the subglottis of injured LTCs compared with controls, with statistical significance at 2, 3, and 4 weeks. Therefore, it is possible that epithelial thickening adds to narrowing of the subglottic lumen after subglottic injury. In fact, when the mean thicknesses of the lamina propria and epithelium of all animals at each posttransplantation time were calculated, a profound difference between experimental and control groups was identified (\(P < .001\)).

In summary, the survival of the grafts, the changes in the subglottic trilaminar layer that are consistent with other models of SGS, and the significant difference between control and experimental LTCs in our study suggest that heterotopic transplanted LTCs in the mouse may be a reasonable model for investigating SGS. This animal model may provide a mechanism for investigating SGS with reduced experimental cost and increased flexibility in experimental manipulation compared with in vivo models of SGS. The ease of generating larger experimental groups is also present because of the improved speed and simplicity with which LTCs can be harvested relative to the undue time restraints and complexity of general anesthesia necessary for live animals. In addition, adjuncts to experimental investigation of disease such as DNA sequencing, molecular reagents, and genetic manipulation are more readily available in the mouse model.

Despite successful survival of the LTC grafts and formation of SGS, drawbacks of the heterotopic model of SGS need to be mentioned. Natural physiologic elements thought to contribute to the development of SGS such as gastroesophageal reflux disease are clearly not present in the transplanted LTC.\textsuperscript{22} Therefore, the development of SGS in the heterotopic model is devoid of clinically relevant variables that affect SGS development in humans. However, this could be seen as a strength of the heterotopic model in that this model isolates for study the intrinsic mucosal physiologic function of the subglottis. Investigating SGS in this controlled situation, excluding natural extrinsic environmental factors, may help improve our understanding of the mucosal and subglottic wound healing process.
CONCLUSIONS

This study describes a novel animal model for the study of SGS. Increases in lamina propria and epithelial thicknesses, greater visualization of extracellular components of the lamina propria, and preservation of the trilaminar structure of the subglottis seen in this animal model are consistent with previously published animal models of SGS. Ease in animal handling, low material costs, flexibility in experimental design, and availability of molecular reagents are benefits of this model for investigating SGS. This animal model may hold promise for future investigations of SGS and mucosal healing.

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