Acute and Chronic Changes in the Subglottis Induced by Graded Carbon Dioxide Laser Injury in the Rabbit Airway

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Objective: To investigate the repair process following carbon dioxide laser injury to the upper airway mucosa (UAM) during the development of chronic subglottic stenosis (SGS).

Design: Animals were assigned to either sham control (cricothyroidotomy only) or injured (cricothyroidotomy and posterior subglottic laser) groups using various carbon dioxide laser exposures (8, 12, and 16 W) for 4 seconds.

Subjects: Twenty-four New Zealand white rabbits.

Interventions: The subglottis was approached via cricothyroidotomy. Sham control airways were immediately closed, whereas injured airways were subjected to graded carbon dioxide laser exposures prior to closure. Airways were endoscopically monitored preoperatively, postoperatively, and on postoperative days 7, 14, 28, 42, 56, 70, and 84. Animals were killed at 14 and 84 days. Subglottic tissue was harvested for histologic evaluation (reepithelialization, extracellular matrix, vascularity, and inflammation).

Main Outcome Measures: Endoscopic visualization and histologic analysis.

Results: (1) Increases in UAM thickness (up to 5 times the thickness of normal mucosa) were observed but were limited primarily to the lamina propria. The mucosal epithelium regenerated without chronic changes. Focal areas of cartilage repair were encountered acutely after injury and to a greater extent in the chronic phases of repair. (2) Acutely, the thickened lamina propria comprised poorly organized extracellular matrix components and demonstrated increases in blood vessel size and number. (3) Histologic changes present in the acute phase only partially resolved in progression to chronic SGS. Chronic SGS was characterized by thick collagen fiber bundles extending into the remodeled subglottic cartilage.

Conclusions: The carbon dioxide laser induces acute changes to lamina propria architecture and vascularity that persist chronically. Elucidating responsible signaling pathways may facilitate the development of therapeutic agents to prevent or reduce the formation of SGS.


The development of airway stenosis remains a challenging problem to treat and correct. Multiple factors may create, perpetuate, and exacerbate this condition, including gastroesophageal reflux disease, infection, traumatic injury to the airway, surgical trauma, and the use of lasers in the treatment of airway lesions. Lasers are often used in the treatment of pathogenic airway processes but have been shown to induce one of the very conditions they have been used to correct, with several reports describing post–laser treatment stenosis. As a research model, laser injury is particularly useful because it can be better controlled and is more uniformly reproducible compared with other methods of subglottic injury. The effects of exposure, in terms of duration and intensity, and those of both acute and chronic changes sustained by a particular area after induced injury with the laser, are not yet clearly understood.

To our knowledge, our previous work was the first to demonstrate, both on gross and histologic examination, the effects of laser injury of varying intensities and extent of mucosal exposure in an animal airway mucosal injury model. Results indicated a marked initial and limited chronic degree of scar formation, notable changes in lamina propria thickness, but no changes in the perichondrium and cartilage layers. Increased blood vessel size and, to a lesser degree, blood vessel number, were shown—findings not reported in previous studies. Based on these results, the initial use of the carbon dioxide laser in the airway using clinically relevant settings was thought to induce a clinically acceptable, limited scar or stenosis, without substantial alterations in the macro-
scoposcopic airway or in the framework anatomy. This initial work was designed to approximate the short-term clinical surveillance undertaken by surgeons following laser interventions in the upper airway and to further describe this as an experimental model. For these studies to be relevant clinically and for the model to truly be applicable to subglottic stenosis (SGS), a chronic wound healing outcome, they must include longer-term postoperative surveillance and follow-up. This is especially important when one considers that a substantial percentage of upper airway interventions occurs in the pediatric population. As such, it is essential to understand and characterize the short- and long-term aspects of upper airway repair following laser injury. Importantly, this understanding must also be obtained in the context of a growing airway, which more closely approximates the pediatric larynx and subglottis.

Our goal in this study was to determine the long-term effects of laser use in the upper airway and to contrast them with short-term outcomes to improve our understanding of the temporal sequence of wound healing processes in the upper airway. The rabbit model was chosen because it is a proven model of airway repair and because it allows us to conduct studies of longer duration of upper airway repair in the context of airway growth, providing an essential correlate to pediatric airways and their growth over time. We believe that our findings help to not only elucidate the mucosal response to laser thermal injury but also to improve our understanding of both the early and late phases of upper airway repair and remodeling with specific emphasis on changes in the lamina propria and cartilage layers.

METHODS

ANIMALS

For this study, the adult New Zealand white rabbit was selected. This animal model has been used extensively in our laboratory and by others in the study of subglottic injury and repair. Two distinct experiments were performed. The first consisted of 10 animals divided into categories detailed in the next subsection, with an intended end point of 14 days after injury. The second consisted of 14 animals, with an intended end point of 84 days after injury.

AIRWAY WOUNDING AND VISUALIZATION

All animal experiments were conducted under approved protocols compliant with Children's Hospital of Pittsburgh (Pittsburgh, Pennsylvania) institutional animal care and use committee regulations. The surgical approach has been previously described. To summarize, each animal underwent a preoperative direct laryngoscopy and video-endoscopy of the subglottis and upper trachea. Through a vertical midline neck incision, the cervical strap muscles were identified and retracted laterally to expose the laryngotracheal complex. A cricothyroidotomy was performed to expose the posterior subglottis.

The airways were then injured as follows. Sham control airways were video-photographed as described herein and converted into digital movie files and images (Pinnacle Systems Software, Mountain View, California). Airways were endoscopically examined on postoperative days (PODs) 1, 7, and 14 for the acute experiment and at PODs 1, 7, 14, 28, 42, 56, 70, and 84 for the chronic experiment. Airways were video-photographed as described herein and converted into digital movie files and images (Pinnacle Systems Software, Mountain View, California).

RESULTS

CLINICAL OBSERVATIONS

Animals were killed by initiating deep anesthesia with a ketamine hydrochloride-xylazine hydrochloride mixture (35 mg/kg and 5 mg/kg, respectively), followed by intracardiac administration of pentobarbital sodium (50 mg/kg). The larynx and proximal trachea were removed by dissection and processed for histologic analysis.

MICROSCOPIC ANALYSIS

Tissue specimens were fixed (10% buffered formalin), paraffin embedded, and sectioned into 5-µm sections. Sections were stained with hematoxylin-eosin to ascertain the following histologic features: cellularity, vascularity, and overall morphologic traits of the epithelial, lamina propria, and cartilage layers. Additional sections were stained with Masson trichrome stain to assess the collagen content of the lamina propria and cartilage layers. Finally, slides were stained with picrosirius red and visualized under polarized light to highlight the degree of organized collagen fibers as determined by birefringence. Subsequent quantitative analysis was performed as follows. Slides were visualized under polarized light, and images were captured demonstrating birefringence present in the posterior aspect of the subglottic mucosa. The total amount of birefringence was normalized to the total mucosal area imaged and compared among the various groups. For histologic analysis, 10 animals were used from the acute, 14-day experiment: sham control (3), 8 W (3), and 12 W (4). A total of 12 animals were used from the chronic 84-day experiment: sham control (2), 8 W (3), 12 W (3), and 16 W (4). Only animals that survived to the intended experimental end point were included in morphologic measurements. The thickness of the lamina propria and cartilage layers was measured (Metamorph; Molecule Devices Corp, Downington, Pennsylvania), averaged across the respective experimental groups, and compared using statistical analysis (t test; significance assigned for P < .05).
Endoscopic visualization and gross imaging

Immediately following surgery and on PODs 1, 7, and 14 for the short-term experiment, and on PODs 1, 7, 14, 28, 42, 56, 70, and 84 for the long-term experiment, an endoscopic examination was performed on each animal, allowing serial documentation of wound maturation and scar development. Figure 1 provides representative images from airways exposed to the various experimental conditions perioperatively and at the termination of both the short- and long-term experiments. As expected, sham control airways showed no scarring of the posterior subglottic mucosa. In contrast, the carbon dioxide laser injury resulted in scarring that persisted into the chronic stages of repair (POD 84) and resulted in the formation of a mature scar localized to the posterior airway mucosa. Over the first 2 weeks following injury, the scar began to remodel and remained localized primarily to the posterior half of the subglottis. In the later stages of subglottic repair, persistent intraluminal circumferential contracture and cartilage deformity, although not complete obstruction of the stenotic region, were noted in the carbon dioxide laser–injured airways.

Histologic analysis

Following gross endoscopic evaluation, airways were sectioned and stained with hematoxylin-eosin for general histologic and morphologic characteristics and Masson trichrome stain to highlight the collagen content of the cartilage and lamina propria layers. The 3 areas of particular interest were the posterior subglottic epithelium, lamina propria, and cartilaginous layers. The subglottic morphologic and histologic characteristics were assessed at PODs 14 and 84 with respect to lamina propria and cartilage layer thickness, and assessed in 2 horizontal planes (superior and inferior) separated by approximately 5 mm of vertical space. The first plane contained the superior aspect of the cricoid cartilage posteriorly and the inferior aspect of the thyroid cartilage anteriorly, representing the superior border of the subglottis. The second plane contained the inferior aspect of the cricoid cartilage posteriorly and the beginning of the first tracheal cartilage posterolaterally, constituting the transition point between the subglottis and adjacent upper trachea. These planes provided anatomically relevant superior and inferior borders to our area of interest (the posterior subglottis) and demonstrated the vertical extent of scar or stenosis formation.

Representative samples of carbon dioxide laser–injured airways at PODs 14 (Figure 2A) and 84 (Figure 2B) are shown at both horizontal levels, at low magnification. These images indicate that, although the various layers of the posterior subglottis are altered following laser-induced injury, the overall anatomy of the upper airway is largely maintained, with lumen patency and relationships between major anatomical landmarks preserved. Figure 3 contains representative images from sham control (Figure 3A) and laser-injured airways (Figure 3B-D). These higher-magnification images demonstrate (1) an increase in blood vessel size and number and (2) an increase in lamina propria thickness, driven partially by an increase in the collagen content as indicated by trichrome staining (Figure 3B and C). In contrast to our previous work, laser damage was found to extend not only to the epithelial and lamina propria layers but also to the subjacent cartilage layer. At POD 14 a layer of newly formed cartilage was identified at the interface between lamina propria and cartilage (Figure 3D). This cartilage did not have the precise architecture of the native cartilage and lacked a sharply delineated border, which in the case of native cartilage identifies the perichondrium. The neocartilage extends on the luminal side of the native cartilage and forms an irregular interface with the reconstituting lamina propria.

Figure 4 illustrates the changes induced in the subglottic mucosa by laser injury at POD 84, which can be
considered to represent the chronic phase of mucosal repair and subglottic stenosis. Sections were stained with Masson trichrome to highlight the connective tissue changes present in the lamina propria and cartilage layers. As expected, the sham control airways had a lamina propria layer of normal thickness, covered by intact epithelium and supported by a cartilage framework that in the inferior plane consists of the cricoid cartilage and posterior-lateral aspect of the first tracheal ring. In contrast, laser-injured airways contained a thickened lamina propria, which is collagen rich, with thick collagen bundles overlaying the supporting cartilage framework (Figure 4C). In general, the increase in vascularity noticed in the earlier phases of mucosal repair appeared diminished, although large vessels were still found interspersed within the newly formed collagen framework. Most important, the small changes in the cartilage layer identified at POD 14 appeared dramatically magnified at POD 84, with newly deposited cartilage substantially increasing the width of the subglottic cartilage layer, primarily on the luminal side of the native cartilage, and as such contributing to the decrease in overall lumen diameter. Importantly, the newly formed collagen bundles and cartilage layer seemed to be connected, with the cartilage appearing to provide anchor points for collagen-based traction forces (Figure 4D).

Figure 2. Morphologic characteristics in sections stained with hematoxylin-eosin. A, At 14 days after subglottic injury caused by the carbon dioxide laser, there was a substantial increase in lamina propria thickness. B, At 84 days after injury, additional airway derangement can be seen with respect to both localized cartilage damage and repair and overall changes in the aspect of the airway lumen. At both time points, the overall airway anatomy and morphologic characteristics were preserved. The top row for each time point is the superior plane; the bottom row, the inferior plane. Original magnification × 4.
after laser injury (Masson trichrome stain, original magnification ×10). As illustrated in Figure 3, glottic lamina propria in the latter stages of tissue repair were characterized by orange-red birefringence, indicating organized collagen, primarily type I (as indicated by orange-red birefringence), in the posterior subglottic lamina propria in the latter stages of tissue repair (POD 84). Picrosirius red has previously been used to highlight organized collagen fibers using birefringence. In our study, we used picrosirius red to demonstrate the presence of organized collagen, primarily type I (as indicated by orange-red birefringence), in the posterior subglottic lamina propria in the latter stages of tissue repair (POD 84). As illustrated in Figure 5, laser-induced injury to the posterior subglottic mucosa resulted in the deposition of substantial amounts of organized collagen, with organization and orientation dramatically different from that found in native, uninjured tissue. The total amount of organized collagen was notably higher in injured mucosa compared with that of sham control airways (Figure 6).

**MORPHOMETRIC MEASUREMENTS**

Measurements in the superior and inferior horizontal planes were made of the lamina propria and cartilage layers at the most posterior aspect of the airway. These data are summarized in Figure 7. Data are presented as follows. Thickness values (in micrometers) are provided for both planes. Comparisons were made between the thickness of the various layers in sham control airways and that of layers in each of the various injury groups. Statistical significance was determined using t test (P < .05).

At POD 14, the lamina propria thickness was found to have increased proportionally to the amount of thermal energy delivered to the posterior subglottis at the time of injury (Figure 7A). The increase was observed in both horizontal planes, but primarily in the inferior plane, at the site where the laser beam was focused. These data confirm that the scarring phenomenon triggered by the carbon dioxide laser is relatively localized to the area of direct injury. In contrast, the proportional relationship between thermal energy and mucosal thickness started to fall off in the chronic phases of subglottic scarring (POD 84). Although mucosal thickness in injured airways remained greater than in sham control airways, there was more variability in the thickness measurements, perhaps reflecting varying degrees of connective tissue resorption and/or remodeling as part of the scar maturation process.

In contrast to the lamina propria layer, in the acute phases of upper airway repair, there was a small but statistically significant (P < .05) increase in the thickness of the cartilage layer of airways injured with a 12 W laser (Figure 7B). In the chronic phase of repair, this phenomenon was magnified, with dramatic increases in cartilage layer thickness at all 3 laser settings.

**COMMENT**

Although the use of the carbon dioxide laser has grown in popularity for the treatment of different pathogenic airway processes, including recurrent respiratory papillomas, cysts, vascular malformations and hemangiomas, webs, and strictures, the risk of permanent injury to the upper airway and its long-term effects on airway growth have not yet been clearly ascertained. Increased observation of relatively poor wound healing outcomes using laser technology has led to a resurgence of modified “cold steel” techniques with the use of, for example, powered instrumentation. Our goal is to develop a model for investigating the effects of altered laser power intensity and area of exposure on the subglottic epithelium, lamina propria, and cartilage in both the acute and chronic stages of healing following injury.

The rabbit model is appropriate for the study of airway repair because it is inexpensive, reliable, convenient, provides reproducible results, and is well suited for longitudinal studies addressing mucosal repair in the context of airway growth.
white rabbit lifespan is 72 months, with sexual maturity achieved by 8 months. At the initiation of all of our studies, the mean age of the rabbits is 3 months old. The current project used 14- and 84-day time frames, paralleling the corresponding time frame in pediatric patients who develop acquired SGS.

Multiple methods of inducing airway stenosis have been described in animal models, including knife incision, wire brushings, electrocautery, pneumatic drill, hydrochloric acid, or silver nitrate. The carbon dioxide laser was used in this project for several reasons: (1) it is a clinically relevant injury mechanism (as detailed in the introductory paragraphs), (2) the degree of injury is more readily titratable, and (3) it allows for better control of injury extent and depth. A recent study has provided an additional line of support for the utilization of lasers in animal models of SGS. Roh et al demonstrated substantial SGS formation in the rabbit animal model using a diode laser. Their histologic findings largely parallel those of our laboratory, although they did not match the range of power settings and extent of injury of our previous work. Unlike our work, their use of the diode laser resulted in substantial early mortality secondary to acute airway obstruction. These results serve to further demonstrate the need for comprehensive analyses of various laser systems in a prospective manner in an animal model.

As in other tissue types, wound healing in the upper airway represents a well coordinated cascade of events in which the ultimate goal is the restoration of tissue structure and function. In postnatal tissue, this process is imperfect and does not completely recapitulate the initial tissue structure and function. In the airway, this is particularly problematic, and in the growing airway, possibly even more so. The subglottis is particularly vulnerable to excessive scarring or stenosis because in children, it is the narrowest part of the larynx. Previous work in our laboratory using qualitative (histologic) and quantitative (image analysis) assessment has demonstrated that scarring and fibrosis develop after isolated laser exposure, with changes occurring in the lamina propria layer. Predominant changes were observed with respect to 2 parameters: (1) thickness and (2) vascularity of the lamina propria. This is consistent with previous work, which noted that it is in the lamina propria and cartilage layers that reparative, rather than regenerative, processes occur, resulting in increased mucosal thickness and associated luminal narrowing. In contrast to other studies, our work demonstrates a dramatic increase in mucosal vascularity following laser injury.

The present study confirms and extends our previous work with respect to several issues: (1) lamina propria vascularity, (2) lamina propria thickness, (3) cartilage damage and repair, and (4) inflammation. With respect to lamina propria vascularity, we again noted a substantial increase in mucosal vascularity with respect to vessel size and number in the acute phase of mucosal repair. As expected, this increased vascularity was diminished in the chronic phases of mucosal repair. When healthy vascular structures are damaged, revascularization is crucial to a timely reparative process. As is the case with other aspects of connective tissue healing, new vessel formation is often poorly organized and excessive. This might represent an increased metabolic demand during mucosal repair, necessitating increased oxygen and nu-
trient supply. The partial resorption of excessive vascularity in the chronic stages of SGS raises important questions with respect to (1) the regulatory mechanisms controlling neovascularization in the upper airway mucosa and (2) the potential of exogenous manipulation of angiogenesis during SGS formation. Currently, we are designing additional prospective, longitudinal studies aimed at further elucidating multiple aspects of mucosal repair, including vascularization. 

Current data indicate that (1) changes in lamina propria thickness persist into the chronic stage of SGS and (2) the mucosal repair process eventually terminates with substantial collagen fiber deposition and reorganization. Of particular interest is the manner in which the collagen matrix is deposited. Unlike the thin fibrils observed in the uninjured mucosa, the extracellular matrix of subglottic stenosis tissue comprises a dense irregular connective tissue of thick, intertwined collagen fiber bundles, reminiscent of collagen deposition during hypertrophic scar and keloid formation.47 Although previous work did not detect substantial changes in the cartilage layer following laser injury, the present study supports our premise that perichondrial penetration is associated with very dramatic changes in the morphologic characteristics of the subglottic cartilage. This is in agreement with published work suggesting that a full-thickness injury would induce a severe cicatricial stenosis, or at least local cartilage frame-deformation.9,22,31,34,42,48 Interestingly, at 14 days after injury, cartilage damage and repair can be detected in relatively localized regions, without a dramatic effect on overall cartilage morphologic traits or luminal dimensions. However, after 84 days, extensive cartilage repair and remodeling were detected, with new cartilage-like material being deposited throughout the posterior aspect of the airway. This suggests that even though certain laser injuries seem to cause minimal mucosal trauma in the acute setting, the thermal energy delivered may be sufficient to trigger profound changes in the underlying cartilage layer over a longer time frame. It also points out that the deeper injury caused by carbon dioxide laser involves at least the perichondrium. Interestingly, the newly formed cartilage appeared to form what could be termed “anchoring points” for the collagenous SGS extracellular matrix. This poses important and possibly troubling questions for further airway growth and maturation. The organization of the normal airway mucosa consists of epithelium, lamina propria, perichondrium, and cartilage. Histologic analysis of these structures in this and other studies indicates a relatively parallel arrangement with apposition of the various layers. This is in stark contrast to the histologic findings of the laser-injured mucosa in the chronic stages of SGS. Collagenous fibers that insert perpendicularly into remodeled collagen provide artificial anchor points for fi-
broblast-mediated contraction and remodeling of connective tissue. These forces, which have been described in other tissue types as being a powerful remodeling tool during wound healing, could begin to markedly alter the normal luminal morphologic traits and impede appropriate airway growth. Particularly troubling is the presence of thick, organized collagen fiber bundles demonstrated by trichrome and picrosirius red staining. This represents the type of mature collagen structure normally associated with other scarring phenomena, such as hypertrophic scars and keloids. This collagenous structure is unlikely to spontaneously regress and may predict permanent structural or morphologic abnormalities.

Connective tissue repair and remodeling is thought to be greatly influenced by the inflammatory component of wound healing. The influx of neutrophils, macrophages, and lymphocytes liberates a wide variety of soluble mediators that influence subsequent fibroblast, epithelial, and endothelial cell activities in the wound bed. In our model, laser injury triggers a notable but transient inflammatory reaction that does not persist into the chronic stages of SGS development. This acute inflammatory reaction does not seem to be sufficient to cause clinically significant airway impedance, in contrast to that triggered by chemical cautery with silver nitrate. We speculate that this is, in part, a result of the fact that once the thermal injury of a laser is imparted, there is no ongoing proinflammatory source. In contrast, certain chemicals, such as silver nitrate, leach into the tissue and likely continue to evoke a host inflammatory response as a “foreign body.” Subsequent study will begin to address the precise composition of the inflammatory infiltrate, as well as its precise temporal profile during SGS development. Together with vasculogenesis, inflammation represents an ideal target for exogenous pharmacologic manipulation aimed at minimizing the extent of scarring and stenosis subsequent to airway injury. We are currently examining the signaling pathways involved in the mucosal inflammatory response to subglottic injury to provide potential targets for pharmacologic and for biologic manipulation of SGS development in the clinical setting.

In conclusion, this study extends our previous work examining the repair processes underlying SGS formation subsequent to mucosal injury. Our current data offer important insights into both the basic research and clinical aspects of SGS development. From a research perspective, they demonstrate the transient nature of inflammation and vasculogenesis and highlight the interaction between these and other more permanent processes, such as lamina propria collagen deposition and cartilage remodeling. From a clinical perspective, our findings lend themselves to some potentially concerning interpretations. What may seem to be a mild thermal injury, localized primarily to the subglottic mucosa, may indeed become associated with a more extensive reorganization of not only the subglottic connective tissue but the underlying cartilage as well. As such, although the injury may be relatively localized, it would not be unreasonable to expect that cartilaginous changes may indeed affect the normal functioning of the structure immediately superior to the injured mucosa, the larynx. In addition, should the “anchor points” described herein prove to be functional anchoring points for fibroblast contraction of the mucosa and underlying cartilage, it may prove to be quite disruptive to airway growth and maturation. These issues will require additional investigation in subsequent studies, with a focus on specific signaling pathways involved in individual aspects of the wound healing process.

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REFERENCES


Correction

Error in Byline. In the Original Article by Otteson et al titled “Acute and Chronic Changes in the Subglottis Induced by Graded Carbon Dioxide Laser Injury in the Rabbit Airway,” published in the July 2008 issue of the Archives (2008;134[7]:694-702), the academic degree of one of the authors is incorrect. The author’s name and degree are Gregory M. DiSilvio, BS.