Cyclooxygenase-2 Inhibition for the Prevention of Subglottic Stenosis

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Objective: To evaluate the role of targeted cyclooxygenase-2 inhibition in reducing scarring associated with a subglottic airway mucosal injury.

Design: Thirty-four New Zealand white rabbits underwent anterior cricothyroidotomy. Subglottic stenosis (SGS) was created by carbon dioxide laser injury.

Intervention: Treatment consisted of intraperitoneal injection of celecoxib or vehicle for 4 days. Endoscopies were performed to assess injury and healing. Subglottic mucosal secretions were collected with Gelfoam swabs (Pfizer Inc) before and after injury and at subsequent time points. Animals were humanely killed at 3 or 8 weeks after injury and airways were excised, followed by gross examination and histologic analysis to assess the severity of SGS. Secretions were analyzed for interleukin-1β, prostaglandin E2 (PGE2), and matrix metalloproteinase-8 by enzyme-linked immunosorbent assays.

Results: Endoscopy showed mild to moderate stenosis in the celecoxib group, but mild to severe stenosis in the vehicle group. Histologic assessment confirmed and quantified reduction in stenosis and scarring as well as advanced reepithelialization. In the healing tissue, mucosal thickening (stenosis) was reduced significantly (P = .02) in celecoxib-treated animals compared with those treated with vehicle, at 3 and 8 weeks (decrease in thickness by 32% and 49%, respectively). Collagen density (fibrosis) was also reduced 25% at both 3 and 8 weeks but the difference was not statistically significant (P = .20). Reduced level of PGE2 in the subglottic mucosal secretions was correlated with mucosal thickness at 8 weeks (P = .02).

Conclusion: Short-duration, anti-inflammatory therapy resulted in reduced stenosis and fibrosis with correlation of PGE2 levels in subglottic mucosal secretions.


The clinical condition of subglottic stenosis (SGS) is initiated by airway mucosal injury that results in acute inflammation and initiation of a healing pathway in which connective tissue wound repair leads to scar formation that may extend to also involve the cartilaginous framework of the cricoid. In the subglottic airway, the end result of this aberrant wound healing response is cicatricial contraction of the lumen, which may significantly limit airflow and ultimately cause life-threatening airway obstruction.

Management of SGS is mainly surgical in nature.1 Recent trends toward minimally invasive endoscopic techniques have evolved in selected patients, resulting in less operative pain, shortened hospitalization, fewer complications, and improved voice outcome.2 Unfortunately, these minimally invasive strategies are curative only in lower grades of stenosis in less severely affected patients. In more severe stenosis, the role of these techniques is relegated to adjunctive amelioration of the degree of airway compromise as a means of simplifying the surgical reconstruction that will ultimately be needed to achieve cure. Despite advanced treatment with combined therapeutic modalities, some children never undergo decannulation and remain dependent on a tracheotomy.

Clearly, the next important advance in the treatment of these children will emerge only from a better understanding and improvement in the aberrant wound-healing response of the injured airway. Inflammation has been established as a determining factor in wound healing, scar formation, and ultimate healing outcome. Interleukin 1β (IL-1β), an early proinflammatory cytokine, activates several inflammatory signaling pathways, including the prostaglandins, such as prostaglandin E2 (PGE2),3 a key product of the arachidonic acid cascade synthesized by cyclooxygenase 2 (COX-2), which has a dual role in inflammation, and fibrotic responses during tissue repair.3

We have previously shown that subglottic mucosal injury upregulates IL-1β and COX-2 messenger RNA (mRNA) and protein levels in tissue as well as mucosal secretions. This early upregulation subdued by postoperative day (POD) 21 of the normal healing process.5

We hypothesized that early inhibition of COX-2 activity will dampen the inflam-
matory cascade and reduce scar formation in the subglottic mucosa. To test this hypothesis, we used our previously established rabbit model of SGS to evaluate the acute and chronic effects of COX-2 inhibition on the development of stenosis.

## METHODS

### ANIMALS

A total of 34 New Zealand white rabbits (Myrtle's Rabbitry Inc and Covance) were used. The experimental injury was created with a carbon dioxide laser as previously described, and the animals were followed for 3 or 8 weeks to assess the intermediate and mature stages of healing.

### AIRWAY WOUNDING AND VISUALIZATION

All animal experiments were conducted under approved protocols compliant with University of Pittsburgh, Children's Hospital of Pittsburgh of the University of Pittsburgh Medical Center (UPMC) (Pittsburgh, Pennsylvania) institutional animal care and use committee regulations, in compliance with federal regulations for humane use of animals in research. The surgery was performed under general anesthesia with a ketamine-xylazine mixture (35 mg/kg and 5 mg/kg, respectively). Briefly, each animal underwent preoperative direct laryngoscopy and video-endoscopy (with a 0°, 3-mm rigid telescope; Karl Storz Inc) of the subglottis and upper trachea. The posterolateral subglottic mucosa was approached via a vertical cricothyrotomy and injured using a carbon dioxide laser (12 W, 1-second pulse, 2 mm of beam diameter at 4 points to create an arc of injury of approximately 90°). Airways were endoscopically visualized on PODs 1, 2, 3, 7, and 14 and at the end points of the experiment (3 or 8 weeks).

### TREATMENT

Animals in the experimental groups were treated systemically via intraperitoneal injection, on PODs 0, 1, 2, and 3, with a COX-2 inhibitor, celecoxib (Celebrex; Pfizer Inc), 3 mg/kg in dimethyl sulfoxide (100 mg/1 mL), while control animals received the vehicle (dimethyl sulfoxide) only. The manufacturer's recommended dosage of celecoxib for children with rheumatoid arthritis older than 2 years is 2 to 5 mg/kg; therefore, the dosage used in this study, 3 mg/kg, is within the therapeutic range.

### SECRETION COLLECTION AND ANALYSIS

Subglottic mucosal secretions were collected from the subglottis preoperatively and on PODs 1, 2, 3, 7, and 14, and either 3 or 8 weeks immediately before the rabbits were killed. Secretions were collected with gelatin foam sponge (Gelfoam; Pfizer Inc) swabs, as previously described. Inflammatory markers PGE2 and IL-1β and fibrotic healing marker matrix metalloproteinase-8 (MMP8) were measured in the secretions using human enzyme-linked immunosorbent assay kits because of the cross-reactivity of the kit antibodies with rabbit antigens (R&D Systems), following the supplier's recommended protocols for each. Levels of these mediators were standardized to secretion weights.

### TISSUE ANALYSIS

At the end of the 3-week and 8-week experiments, animals were killed by initiating deep anesthesia with a ketamine-xylazine mixture (35 mg/kg, and 5 mg/kg, respectively), followed by intravenous administration of pentobarbital sodium (50 mg/kg). The airway sections encompassing the larynx and proximal trachea were removed by dissection and fixed in 10% buffered formalin.

### MACROSCOPIC AND MICROSCOPIC ANALYSIS

The excised airways were examined and grossly dissected in the region of the injury and photographed under a dissecting microscope. All samples were then processed for paraffin embedding and thin sectioning (5-µm sections). Sections were mounted on glass slides and stained with hematoxylin-eosin to determine the overall morphologic character of the epithelium, lamina propria, and cartilage layers. Sequential serial sections were stained with Masson trichrome stain to emphasize the connective tissue of the lamina propria and cartilage layers. The thickness of the mucosa overlying the cricoid cartilage mid-posteroanterior direction was measured with MetaMorph imaging software (version 7.0; Molecule Devices Corp), and the mean reduction of the thickness was determined as percentage comparison with injured, vehicle-treated control animals. The next sequential serial sections were stained with picrosirius red and viewed under polarized light to visualize the birefringence of collagen fibers, which is largely due to co-aligned fibrils of type I and type III collagens, as a measure of maturity and density of the healing connective tissue. Images were taken under polarized light and quantified, using MetaMorph software for the percentage of the area occupied by birefringent fibril bundles in the region of injured mucosa.

### STATISTICAL ANALYSIS

Wilcoxon rank-sum test was used to compare the mucosal thickness, percentage of the area threshold of light objects, and biomarker levels between the 2 groups. The Spearman rank correlation was used to quantify the correlation between specific marker level and clinical outcomes (ie, the mucosal thickness and the percentage of the area threshold of birefringent collagen fibrils).

### RESULTS

#### CLINICAL OBSERVATIONS

There were 5 animal deaths. Of these, 3 animals were in the vehicle-treated group at POD 1, and 2 animals were in the celecoxib-treated group at PODs 11 and 22. One vehicle-treated animal was killed on POD 2 because of a surgical complication unrelated to the subglottic injury. One of the celecoxib-treated animals from the 3-week group was excluded because of granulomatous tissue at the skin incision line extending to the lumen. Mild to moderate grades of respiratory effort were observed primarily in the vehicle treatment group, especially on PODs 1 to 4. There was no mortality at time points correlating with acute SGS (PODs 5-9) in celecoxib-treated animals.

### HISTOLOGIC ANALYSIS

#### Three-Week Experiment

Airways were examined, and cross-sectional areas of injury were imaged (Figure 1 A and B). The results showed gross luminal narrowing and thickened mucosa, most prominently in the injured posterior subglottis.
Sections were stained with Masson trichrome to highlight the increased collagen content of the cartilage and the lamina propria layers (Figure 1C and D). Vehicle-treated specimens showed thickened lamina propria at the posterolateral walls with mucosal and epithelial disruption and more involvement of cartilage compared with celecoxib-treated specimens. The celecoxib-treated group had less pronounced lamina propria thickening and near-complete reepithelialization. The reduction of subglottic mucosal thickness seen with the COX-2 inhibitor was variable individually (Figure 1E-H) (Table).

Celecoxib treatment reduced the collagen deposition compared with vehicle, as shown in picrosirius red-stained sections. The normal lamina propria of the subglottic mucosa is thin and lacks fibrous bundles, but following injury, the lamina propria displayed thick, prominent collagen fibril bundles. Well-aligned, organized fibers were seen with vehicle alone, indicating fibrosis of the mucosal layer (note that uninjured control mucosa adjacent to injury site is thin, with very few collagen fibers). By comparison, the celecoxib-treated airways showed measurably less mucosal thickening and reduced fibrosis (Figure 1I and J).

### Table. Posterolateral Subglottic Mucosal Thickness Following Injury

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<sup>a</sup>There were more animals in the celecoxib treatment groups.

Cross-sectional images showed thickened subglottic mucosa over the cricoid cartilage (Figure 2A and B). There was a sustained reduction of severity of stenosis with celecoxib treatment (Figure 2C and D), even though short courses of treatment were administered only during the early phase of healing.

**Eight-Week Experiment**

Microscopic examination of the posterolateral mucosa in the vehicle-treated group consistently showed disrupted epithelium, thickened lamina propria, and cartilage involvement, whereas while the celecoxib-treated airways had less lamina propria thickening, almost complete reepithelialization, and less fibrosis (Figure 2E-H).

Similar to the 3-week experiment, picrosirius red staining showed well-aligned collagen fibrils with vehicle treatment alone, whereas celecoxib treatment reduced collagen deposition (Figure 2I and J).
Combined Analysis of Both Experiments

In the region of injury, the posterior airway mucosal thickness was less in the celecoxib-treated group vs the vehicle-treated group at 3 weeks ($P = .14$) and 8 weeks ($P = .08$) (32% and 49%, respectively). The combined analysis of both study groups showed significant reduction of stenosis with treatment ($P = .02$) (Figure 3). Similarly, fibrosis, as measured by abundance of collagen fibrils, was also reduced in celecoxib-treated wounds vs vehicle-treated wounds (Figure 4). Although celecoxib treatment showed a 25% reduction compared with vehicle at each of the time points, this difference was not statistically significant ($P = .20$).

**Inflammatory Mediators in Secretions**

Laser injury caused acute induction of PGE2 levels in rabbit airway secretions in the vehicle group at POD 1, which remained elevated in the first 3 PODs and showed peaks at PODs 7 and 21 followed by a decline at POD 56, which was still higher than acute induced levels. The celecoxib-treated group exhibited lower induction levels by comparison (Figure 5), as expected, since COX-2 activity is responsible for synthesis of PGE2 during acute inflammation.

Similarly, IL-1β levels in secretions were higher in the early postoperative period, followed by a more rapid de-
cline, unlike PGE2, starting from POD 7 in both treatment groups. Although IL-1\(\beta\) levels seemed to be reduced with celecoxib treatment at POD 3 when compared with vehicle, the difference was not statistically significant (Figure 6).

The level of MMP8 showed a gradual increase with time starting from POD 3 in both treatments and reaching a peak level at POD 21. At the end point of the experiment (POD 56), despite some decline, levels were still elevated compared with baseline values. Celecoxib treatment had no measurable influence on MMP8 levels in subglottic airway secretions (Figure 7).

Considering the composite of responses for each animal in both the vehicle- and celecoxib-treated groups, the degree of stenosis measured by mucosal thickness correlated with the individual PGE2 levels in secretions at POD 3 (\(P = .02\)) (Figure 8). In contrast, there was weak correlation with levels of IL-1\(\beta\) and correlation with levels of MMP8 at any time point (plots are not shown).

COMMENT

The incidence of SGS in infants and children significantly increased as neonatal intensive care improved to the point where many of these infants survived, in part because of prolonged endotracheal intubation.\(^\text{10}\) Although further improvements in airway management in neonatal intensive care units have reduced the incidence of clinically apparent SGS,\(^\text{11}\) it remains a challenging entity to treat. At one major pediatric hospital in the United Kingdom, the incidence of SGS was found to be 4.95 per 100,000 live births from 1993 through 2003.\(^\text{12}\) Acquired SGS remains one of the most common causes of stridor and airway obstruction in infants and the most common tracheolaryngeal abnormality requiring airway intervention.\(^\text{13}\)

Clinically significant SGS is more likely to be encountered in pediatric patients since it has been traditionally believed that the subglottis represents the narrowest cross-sectional segment of the pediatric airway.\(^\text{14}\) Recent reports have challenged this dictum, contending instead that, similar to adults, the glottis is the narrowest segment of the larynx in infants and children.\(^\text{15}\) Nonetheless, experts agree...
that the mucosa and cartilaginous framework (the cricoid ring) of the subglottis is most vulnerable to injury and subsequent stenosis from prolonged endotracheal intubation compared with the pliable glottis regardless of which region is actually flow-limiting in the normal state. This is especially true in the pediatric age group and is borne out in clinical experience.16

Despite a wide variety of surgical approaches, the management of SGS remains a formidable challenge for both physician and patient. Additional important advances in the outcome of SGS will derive from an understanding of the cellular and molecular processes underlying the development of SGS following airway injury.

As previously demonstrated, activation of inflammatory pathways is an important component of airway mucosal scarring and fibrosis.5,7 In other tissue types, modulation of inflammation has been linked to decreased scarring and fibrosis.17 Although some degree of inflammation is necessary for reparative postnatal skin wound healing, regenerative "scarless" fetal skin wound healing ensues in the absence of inflammation.18 Therefore, downregulation of inflammation is likely beneficial for reducing scarring and promoting regenerative (vs reparative) healing. COX-2 mRNA and protein levels are higher in the late gestational period, when scarless healing switches to fibrotic repair, suggesting the involvement of the COX-2 pathway in scar production.19

In this study, we used an established carbon dioxide laser–induced SGS model6 to investigate the effect of short-term COX-2 inhibition on acute and long-term mucosal healing in subglottic airway.

Histologic tissue analysis demonstrated improved mucosal healing, with marked reduction of scar formation, more complete reepithelialization, and diminished stenosis severity with celecoxib treatment. Taken together with histologic improvement and the lack of acute mortality with relatively short treatment duration, the use of a COX-2 inhibitor presents an appealing prophylactic and therapeutic option for SGS.

Because COX-2 is responsible for the synthesis of PGE2, it is expected that the levels of this mediator should be lower in the secretions of celecoxib-treated animals (Figure 5). Vehicle-treated control animals showed a bimodal pattern of PGE2 subglottic secretory levels over time, consistent with findings in our previous studies5,7 and reflecting the involvement of PGE2 in the acute inflammatory and the tissue-remodeling phases of wound healing. The correlation between PGE2 level in the secretions and corresponding mucosal thickness at POD 56 supports the relevance of PGE2 as a marker for degree of stenosis (Figure 8).

Although upregulation of IL-1β was marked after injury in subglottic secretions, it was not significantly affected by COX-2 inhibition, which is not surprising given that it is upstream of PGE2 inflammatory signaling (Figure 6). IL-1β increases mucus secretion directly by COX-2 induction and PGE2 release in human airway epithelium,20 and indirectly by activation of CD4⁺ cells and by inflammatory cell recruitment via upregulation of expression of cell surface adhesion molecules (intercellular adhesion molecule-1 [ICAM-1], vascular cell adhesion molecule 1 [VCAM-1]) on vascular endothelium.21 On PODs 1 to 3, endoscopic examination indicated luminal edema and induction of secretions in both groups. Acute mortality on POD 1 in the vehicle- treated group, but not celecoxib-treated group, might be correlated with a beneficial effect of COX-2 inhibition of mucus production by upregulated IL-1β. IL-1β levels at POD 3 showed a weak correlation with mucosal thickness at POD 56, which we attribute in part to the high variability of the samples at this time point (and which might be overcome with increased sample size).

MMP8 (collagenase-2 or neutrophil collagenase), one of the zinc-dependent endoproteases, in particular, has been shown to play a prominent role in healing of mucosa of the respiratory tract, with higher levels detectable in mucosal secretions being associated with airway fibrosis.22

Herein, we showed initially decreased levels of MMP8 in the acute period of mucosal healing and gradually increasing concentrations similar to the pattern of expression observed in human dermal wound exudates in vivo.23 The peak level of MMP8 at POD 21 in subglottic secretions is representative of the chronic nature of SGS. MMP8 levels in the secretions did not correlate with mucosal thickness and did not show a reduction with celecoxib treatment (Figure 7), indicating that the reduction in mucosal thickness by celecoxib treatment was unrelated to decreased MMP8 activity in the wounds. However, MMP8 activity is only 1 contributor to fibrotic healing, so future studies should include other metalloproteinases and tissue inhibitors, as well as other mediators of fibrotic healing to give a better understanding of the pathogenesis of SGS.

In wound healing and scarring, preventive strategies are nearly always preferable to the need for therapeutic interventions after fibrotic healing has occurred. COX-2 inhibitors have been widely used as anti-inflammatory treatment and cancer prevention despite certain known adverse gastrointestinal and cardiovascular effects with prolonged therapy.24 There are conflicting results in regard to cardiovascular adverse effects, and there is a need for long-term follow-up studies. Judicious use of COX-2 inhibitors may
maximize the therapeutic benefits while minimizing adverse effects, hence resulting in a favorable safety therapeutic index. While celecoxib treatment did not completely prevent mucosal thickening and fibrosis, both were reduced, which may be highly relevant in clinical applications, since a reduced degree of stenosis may be a successful outcome with respect to airway patency and avoiding the need for surgical intervention. Going forward, the moderate dose and relatively short duration of COX-2 inhibition used in this study heighten optimism that an effective dose and duration of treatment can be determined that will minimize the risk of adverse treatment effects to acceptable levels, even for pediatric patients. In contrast, topical delivery of a COX-2 inhibitor was only marginally effective in reducing the degree of SGS in our preliminary animal experiments; however, better outcomes may be achievable with adjustments to the dosing regimen and method of topical delivery, further reducing concerns about adverse effects with systemic administration (data not shown).

In conclusion, low-dose systemic, short-course, specific anti-inflammatory therapy resulted in promising macroscopic reduction of subglottic stenosis and microscopic reduction of fibrosis.

Use of airway secretions to measure key markers of injury, inflammation, and healing is a relatively noninvasive and feasible method of predicting SGS outcome. Establishing surrogate markers of airway injury outcome would have a considerable impact on the clinical management of pediatric critical airways with regard to indicating the need for anti-inflammatory medications, downsizing the endotracheal tube size, or performing tracheotomy in lieu of endotracheal intubation. Currently, there are no tests to aid in these clinical decisions, which are often made randomly and subjectively with little evidence basis to support them. Future studies are needed to assess efficacy in a larger trial while evaluating different COX-2 inhibitor dosing and treatment duration algorithms targeted for clinical applications.

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