Mucosal Wound Healing in a Rabbit Model of Subglottic Stenosis

Biochemical Analysis of Secretions

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**Objectives:** To build on work in laryngology and oral biology that suggests utility in the assay of secretions collected from wound sites as a predictive instrument to determine which infants will likely develop subglottic stenosis following endotracheal intubation and to document and describe the wound-healing process.

**Design:** Prospective, randomized design.

**Intervention:** Laser-induced subglottic injury was established in 3 rabbits. Secretions were collected from the subglottic region at 6 time points from days 4 to 21 following injury and from 4 uninjured control airways. The secretions were then subjected to enzyme-linked immunosassays for interleukin 1β and prostaglandin E2.

**Subjects:** Three adult New Zealand white rabbits.

**Main Outcome Measures:** Interleukin 1β and prostaglandin E2 levels.

**Results:** Measurable amounts of both mediators were obtained. Furthermore, different temporal patterns of expression were observed with interleukin 1β, showing increased levels on days 4 to 18, and with prostaglandin E2, showing increased levels on days 7 to 18. These results concur with emerging data regarding the role of each mediator in the wound-healing process.

**Conclusion:** Although in its infancy, the analysis of secretions collected from the site of injury in the subglottis may have utility in the management of patients following intubation-related trauma.


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**ENDOTRACHEAL INTUBATION** in the neonatal population is an increasingly common practice for the establishment of a safe airway and ventilatory support. Given that the subglottis is the narrowest part of the laryngotracheal region in infants and children, intubation-related trauma can result in subglottic stenosis (SGS), a potentially fatal narrowing of the airway lumen. Clinically, the ability to predict which infants will develop SGS based on the appearance of acute injury is limited. A marker profile providing prognostic information would assist clinicians considerably in determining the need for close observation and management of patients at risk for developing SGS, who potentially require intervention. To that end, the present study seeks to describe the profile of 2 key inflammatory mediators in secretions from the site of injury as potential predictive indicators of stenosis. Although this study is preliminary, it seeks to determine the potential feasibility for a noninvasive assay to detect mediators of injury, inflammation, and tissue repair in secretions collected from the subglottis as potential indicators, or markers, of future stenosis yielding critical clinical information that may direct treatment in the at-risk population.

The clinical utility of measuring biochemical markers in secretions has been demonstrated in several types of mucosal injury. Predominantly, the assay of secretions has been used in the dental/oral biology literature. Elevated interleukin 1β (IL-1β) levels in gingival crevicular fluid signify the presence of periodontal disease, as well as yield critical information regarding the severity of the disease. In addition, lung and gastric lavage fluids have been analyzed for the presence of factors affecting healing and fibrosis to document the wound-healing response, further confirming the emerging potential of such assays. Our laboratory has extended secretion analysis to wound healing in the upper airway. Secretions collected from the surface of the vocal folds in human subjects have been used to describe the acute inflammatory response fol-
lowing heavy vocal use. In addition, we have reported that patients with vocal fold lesions present with differing profiles of wound-healing markers compared with normal subjects. We have also used an animal model to describe the temporal expression of such markers associated with vocal fold wound healing.

Markers of acute inflammation have been thoroughly described in the literature. These markers are primarily involved in the host response to injury or infection and are typically classified as either proinflammatory or anti-inflammatory. It has been hypothesized that the relative susceptibility to disease may be determined by the balance of expression between proinflammatory and anti-inflammatory cytokines. Furthermore, it is hypothesized that those patients who develop stenosis of the airway will present with a distinctly different inflammatory response and, therefore, profile of inflammatory mediators.

Numerous steps must be taken in the hope of developing a prognostic tool. The present study sought to tackle the obvious question of feasibility, as well as differential expression of inflammatory mediators in secretions. Furthermore, the selection of mediators is critical. This study examined IL-1β and prostaglandin E2 (PGE2) concentrations in secretions. Interleukin 1β is an obvious target for such an experiment because its role is well defined as an early mediator of the inflammatory response. A lipid-based mediator, PGE2 has a more ubiquitous role in wound healing and may be expressed in both early and later stages of wound healing. Prostaglandin E2 has been implicated in inhibiting profibrotic responses, including collagen production, contraction of extracellular matrix, and fibroblast proliferation. Interestingly, the application of exogenous PGE2 has been shown to stimulate epithelial migration, suggesting its involvement in this phase of the wound-healing response. Both markers were selected owing to their presence in mucosal secretions. In contrast, other mediators such as transforming growth factor β appear to remain tissue bound and are not detected in secretions.

Specifically, the present study addresses the following 3 experimental questions: (1) Can quantifiable amounts of biochemical mediators associated with wound healing be obtained from secretions collected from the site of injury in the subglottis? (2) Is there differential temporal expression of these mediators (IL-1β and PGE2) following subglottic injury? And (3) Does the temporal expression of these mediators correlate with their known functions in the wound-healing process? A positive response to these questions would support the feasibility of using secretion assays to monitor the wound-healing cascade, which would validate future investigation into the potential utility of such assays to determine the likelihood of stenosis in the neonatal population.

The study was approved by the Animal Research and Care Committee of the Children’s Hospital of Pittsburgh, Pittsburgh, Pa. A subglottic injury was induced in 3 adult New Zealand white rabbits. Following open cricoid resection, carbon dioxide laser (four 1-second pulses at 5 W) was used to create the subglottic injury. This procedure was used to create an injury of controlled, uniform depth because we have previously reported that depth of injury correlates with the severity of the resulting stenosis.

Following the creation of the injury, a small piece of Gelfoam (Pharmacia & Upjohn, Kalamazoo, Mich) held by forceps was used to collect secretions from the wound area. The cricoid ring was then placed back into the anatomic position and opposed using a single layer of 5-0 chromic suture in a simple interrupted fashion. The skin was closed using a 4-0 nylon suture in a simple interrupted fashion. All animals were given subcutaneous injections of butorphanol, 0.3 mg/kg, during the immediate postoperative period (48 hours) for analgesia.

**SECRETION COLLECTION**

Secretions were collected from the 3 rabbits with subglottic injury and from 4 uninjured control animals using a modified pediatric laryngoscope to visualize the vocal folds. A narrow tube was placed within the lumen of the laryngoscope and between the vocal folds. A small piece of Gelfoam was placed through the tube, and manipulated on the injury site to ensure the isolated capture of wound secretions. The swab was then removed, placed in a microfuge tube, and stored at −80°C for future analysis. Secretions were collected at 6 time points from the injured animals (4, 7, 12, 15, 18, and 21 days after wounding). These time points were selected to capture the temporal profile of wound-healing events. Earlier time points (1 or several hours after injury) would most likely reflect the acute inflammatory response, as our previous studies demonstrate. However, the present study sought to describe mediator expression during the overall wound-healing process, not limited to the acute inflammatory stage. On review of the data, it was noted that concentrations of both markers were significantly elevated after cricoid resection but before laser injury. This is likely because of the inherent trauma of cricoid resection and the resultant inflammatory response. Furthermore, by 21 days following injury, marker concentrations fell well below the preinjury levels, confirming this phenomenon. Therefore, secretions were collected from 4 uninjured rabbits at a single time point.

**SECRETION EXTRACTION AND ANALYSIS**

The secretions were extracted from the Gelfoam using 500 µL of sterile saline. The tubes were vortexed for approximately 5 seconds and centrifuged at 10000 rpm for 3 minutes. The supernatant solution was collected. This procedure was repeated to yield approximately 1000 µL of supernatant. The entire protocol was performed in a cold temperature to minimize the risk of denaturation at room temperature. As a further control, this procedure was performed on clean Gelfoam to ensure the marker profile is indicative of wound levels. This procedure has been described previously by our group. Interleukin 1β and PGE2 were assayed using enzyme-linked immunosorbent assay kits following the supplier’s recommended protocol for each (R&D Systems, Minneapolis, Minn.). Following standardization, 1-sided t tests were used to assess differences in marker expression between time after injury and the normal/uninjured state.

**RESULTS**

Measurable amounts of both IL-1β and PGE2 were obtained in all samples, although at very low levels in secretions from uninjured airways. Concentrations of IL-1β and PGE2 were standardized based on secretion weight be-
cause this parameter was found to be the most consistent, compared with total volume, which was nearly impossible to measure accurately with our method of collection, or total protein. Previously, our laboratory reported on the utility of the highly sensitive bicinchoninic acid protein assay (Sigma Chemical Co, St. Louis, Mo) as a means to standardize marker concentrations.13 However, such analyses are susceptible to increased error when even a trace amount of blood is present in the sample because protein levels in blood and serum are markedly higher than protein concentrations in mucosal secretory fluids. Trace amounts of serum were present in the early subglottic wounds. Therefore, sample weight was measured as follows. Microfuge tubes were weighed prior to secretion extraction. After extraction, the tube with the Gel-foam swab was placed in a vacuum desiccation chamber for 5 days to remove residual liquid. The tube with the Gel-foam swab was weighed again following desiccation, and the difference in weight before and after extraction and desiccation yielded the weight of the secretion. Concentrations for each biochemical mediator were then divided by the weight of the secretion; all results are reported in picograms per gram of secretion.

INTERLEUKIN 1B

As shown in Figure 1 and Table 1, IL-1β concentrations were upregulated in the immediate postinjury period, starting with day 4, compared with expression in control animals (P = .01). However, as described earlier, levels were drastically increased in the preinjury state, which was likely a result of the inflammatory response to the surgical procedure. Levels remained elevated through day 15 and then began to decrease by day 18. In addition, IL-1β essentially returned to baseline levels by day 21 following injury (the end point of the current study) (P = .16).

PROSTAGLANDIN E2

Similar to IL-1β, PGE2 levels increased shortly after injury as shown in Figure 2 and Table 2. However, statistically significant increases were not obtained until day 7 following injury (P = .046). Prostaglandin E2 levels peaked at day 15 and then began to decrease. As with IL-1β, PGE2 concentrations returned to control levels by day 21 (P = .18).

COMMENT

Similar to findings at other mucosal sites, inflammatory mediators of wound healing are detectable in secretions collected at the site of subglottic injury. This positive response to our initial experimental question is encouraging. Furthermore, the patterns of expression of both mediators vary with time following injury. This is particularly

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Table 1. Mean Interleukin 1β (IL-1β) Concentrations in Secretions From the Site of Subglottic Injury as a Function of Time

<table>
<thead>
<tr>
<th>Condition</th>
<th>IL-1β Concentration, Mean ± SEM, pg/g</th>
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<tbody>
<tr>
<td>Control</td>
<td>7.0 ± 0.3</td>
</tr>
<tr>
<td>4 d</td>
<td>263.1 ± 95.7</td>
</tr>
<tr>
<td>7 d</td>
<td>388.2 ± 167.9</td>
</tr>
<tr>
<td>12 d</td>
<td>270.2 ± 113.2</td>
</tr>
<tr>
<td>15 d</td>
<td>436.5 ± 235.8</td>
</tr>
<tr>
<td>18 d</td>
<td>67.6 ± 29.9</td>
</tr>
<tr>
<td>21 d</td>
<td>24.2 ± 18.7</td>
</tr>
</tbody>
</table>

Table 2. Mean Prostaglandin E2 (PGE2) Concentrations in Secretions From the Site of Subglottic Injury as a Function of Time

<table>
<thead>
<tr>
<th>Condition</th>
<th>PGE2 Concentration, Mean ± SEM, pg/g</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>4420 ± 1041</td>
</tr>
<tr>
<td>4 d</td>
<td>33 497 ± 19 815</td>
</tr>
<tr>
<td>7 d</td>
<td>44 413 ± 23 012</td>
</tr>
<tr>
<td>12 d</td>
<td>71 647 ± 17 869</td>
</tr>
<tr>
<td>15 d</td>
<td>130 580 ± 67 094</td>
</tr>
<tr>
<td>18 d</td>
<td>31 785 ± 13 671</td>
</tr>
<tr>
<td>21 d</td>
<td>15 434 ± 12 747</td>
</tr>
</tbody>
</table>

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interesting because IL-1β and PGE2 have distinct roles in the wound-healing process that appear to be dependent on the temporal characteristics of the wound. Interleukin 1β expression is elevated shortly after injury. This corresponds well with its accepted role in the inflammatory process immediately following injury. In contrast, PGE2 expression did not significantly increase until day 7 following injury. This may reflect its role in later stages of wound healing.

The concentrations of both markers in secretions converge with the emerging literature that suggests a reciprocal relationship between IL-1β and PGE2. The proinflammatory nature of IL-1β has been well described. Interleukin 1β stimulates the expression of proinflammatory genes including type II phospholipase A2, cyclooxygenase-2 (a key enzyme in the synthetic pathway for PGE2), and inducible nitric oxide synthase, as well as other cytokines and chemokines. In contrast, PGE2 is likely a regulator of the inflammatory response with both proinflammatory and anti-inflammatory activities. The proinflammatory activity of PGE2 appears to be related to IL-1 regulation of IL-6 production, another key proinflammatory cytokine. In contrast, investigators have described several of the anti-inflammatory actions associated with PGE2. Prostaglandin E2 can inhibit IL-1β up-regulation of inducible nitric oxide synthase and thereby reduce nitric oxide production. In addition, PGE2 signaling decreases macrophage secretion of both cytokines and chemokines.

The present study appears to confirm this relationship between IL-1β and PGE2, which is further validation of the assay. Maximal expression of PGE2 correlates well with the initiation of decreased IL-1β expression. It is likely that the maximal expression of PGE2 on day 15 following injury marks the transition between the acute inflammatory response and the initiation of neomatrix formation and organization. These findings correspond well with our laboratory’s previous histologic investigation into the wound-healing process in the subglottis. Goldstein et al reported that a subacut infiltrate was present on day 14 following injury. In addition, fibroplasia and fibrosis were present on days 7 to 21 following injury. It is hypothesized that mediator presence in secretions may yield noninvasive information regarding wound-healing events within the tissue. These hypotheses remain conjecture at this point and warrant further investigation. However, the results are promising and may lead to the development of a clinical tool in the future.

Although the present results are encouraging, several potential problems exist. Primarily, the present study used relatively few animals, contributing to increased variance of the data set. Given the preliminary nature of our study and the relative invasiveness of injury creation, minimizing the use of animals was a priority. Despite this variability, clear trends emerged, which are encouraging for future investigation. The differences between the injured sites and control sites are significant, establishing the validity of the assay. Another potential problem is that a “sham” procedure was not used. It is possible that the surgical resection of the cricoid may cause an inflammatory response captured in secretions. Therefore, it is impossible to suggest that the profile of markers is due only to the laser-induced injury and not the surgical procedure as well. However, we contend that if this is the case, the results are still valid in demonstrating increased levels of inflammatory mediators in the secretions following mucosal injury. In addition, the time points chosen were thought to reflect times that were more likely to be clinically relevant with respect to duration of the endotracheal tube. It was hypothesized that measurement at earlier points would yield even higher levels of IL-1β, as were found in our other studies. Now that the methods have been refined, future studies will use more animals, additional time points, and controls to ensure better correlation of marker expression relative to the subglottic injury. Clearly, however, this is an area that warrants further investigation.

The basic feasibility of a noninvasive assay to detect mediators of injury, inflammation, and tissue repair in mucosal secretions has been established, in both the study presented here and in our previous publications. Future work will focus on the correlation of the quantitative and temporal expression of IL-1β, PGE2, and other mediators with the extent of subglottic injury and ultimate healing outcome. As potential indicators, or markers, of future stenosis, concentrations of these mediators would provide critical clinical information and help to direct treatment in the at-risk population.

CONCLUSIONS

Overall, these results are encouraging and will stimulate further investigation. There are strong correlations between mucosal injury and the levels of some inflammatory and wound-healing mediators detected in mucosal secretions. These results indicate the feasibility of noninvasive assessment of injury and healing and provide support for more extensive analyses in the rabbit model as well as future analyses in human subjects. Future studies will target the fibrotic process itself as well as those mediators that may be hallmarks of fibrosis.

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REFERENCES


