Biochemical Markers Associated With Acute Vocal Fold Wound Healing: A Rabbit Model

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Summary: This study seeks to determine the ability of enzyme-linked immunosorbent assays (ELISAs) of vocal fold secretions to detect and describe the acute tissue response to injury in a rabbit vocal fold model. Vocal fold secretions were collected before the induction of a unilateral surgical injury to the vocal fold and at 6 timepoints after injury (1, 5, 7, 10, 14, and 21 days). Secretions were then subjected to ELISAs to assess concentrations of interleukin-1 beta (IL-1β) and prostaglandin-E2 (PGE-2). The results indicate that ELISAs may be useful in documenting fluctuations in these markers associated with the wound healing process in the rabbit model. The temporal expression of both IL-1β and PGE-2 was consistent with their proposed roles in the wound healing cascade in other systems, pointing to the potential that surface secretions may be at least partial indicators of wound healing events within the tissue.

Key Words: Larynx—Vocal folds—I injury—Secretions—Cytokines—Wound healing.

INTRODUCTION

Wound healing in the skin requires a highly organized series of events including hemostasis, inflammation, re-epithelialization, cell proliferation, matrix deposition, angiogenesis, and wound contraction. A general timeline for such events and key mediators of each stage have been described in the skin and result in the resolution of epithelial defects as well as the reconstitution of functional tissue. The long-term sequelae of wound healing in the vocal folds, specifically, the formation of vocal fold scar and the resultant alteration in biomechanical function, have been described. However, the acute response to vocal fold injury has not yet been investigated. Insight into the sequence of events associated with the acute response to injury in the
vocal folds might yield important information about critical periods for intervention as well as prognostic indicators for outcomes of treatment. The current study is a component of a long-term research program in our laboratory addressing the acute wound healing response in the vocal folds, as well as wound healing itself.

A description of the sequence of wound healing events in the skin serves as a foundation for the current study. Initially, after acute injury, the inflammatory response orchestrates the cascade of events associated with wound healing, and it ensures immune competence after injury. The inflammatory response is initiated by the extravasation of blood constituents from damaged vessels. Chemoattractants, cytokines, and growth factors are released that recruit neutrophils and monocytes to the injury site and directly stimulate keratinocytes and fibroblasts to initiate the repair process by assuming the repair phenotype. Macrophages continue to clean and debride the wound, in addition to releasing numerous cytokines key to fibroblast chemotaxis and proliferation. Those cytokines include platelet-derived growth factor (PDGF), fibroblast growth factor (FGF), and transforming growth factor-beta (TGF-β). In addition, macrophage-derived cytokines such as interleukin-4 (IL-4) are responsible for tissue formation (ie, collagen production in fibroblasts).

The re-establishment of a functional epithelium at the site of the wound is a key aspect of wound healing. Clinically, a dermal wound is re-epithelialized when a water-impermeable seal is present over the wound. Keratinocyte migration into the site of injury from the periphery is stimulated by epidermal growth factor and fibroblast growth factor released by both inflammatory cells and fibroblast growth factor. This process typically begins 24 to 48 hours after injury. Wound contraction begins 4 to 5 days after injury. Contraction is characterized by the centripetal movement of the wound edge toward the center of the wound. Both myofibroblasts and fibroblasts are thought to be responsible for this process. Scar remodeling is the final stage of wound healing. The accumulation of collagen becomes stable at approximately 21 days after injury. However, remodeling, involving the synthesis and degradation of collagen, may continue for several months, and it contributes to the gradual regain of tissue tensile strength.

As noted, the sequence of events is relatively well documented in the skin, and little is known about acute wound healing activities in the vocal folds. Because the vocal fold has a unique subepithelial structure and highly specialized biomechanical functions, the study of the processes associated with acute wound healing in the vocal folds is warranted. This issue is addressed in this present study.

Before the introduction of the experimental questions, background information regarding the methods used in the current investigation is relevant. Previous investigation has suggested that analyses of biochemical markers associated with wound healing extracted from secretions collected from the vocal fold surface may serve as a noninvasive means to document the wound healing process in human vocal fold injury. In short, dramatic shifts in pro-inflammatory markers such as interleukin-1β (IL-1β), tumor necrosis factor-alpha, and matrix metalloproteinase-8 were noted in a single adult subject after a 1-hour period of intense vocal loading that induced prominent vocal fold edema and dysphonia. In another study, differential profiles of both IL-1β and PGE-2 were noted in surface secretions of patients with various lesions of the vocal folds. The current study seeks to build on the previous ones by investigating the time-dependent expression of key biochemical markers in vocal fold secretions acutely after vocal fold injury, and to assess the extent to which such expression might reflect processes within the tissue. To that end, two biochemical markers were selected based on their known temporal regulation in the wound healing response in other tissues: interleukin-1β (IL-1β) and prostaglandin-E2 (PGE-2).

Interleukin-1β is a prototypical pro-inflammatory cytokine. It is the secreted isoform of the IL-1 family. It is produced by macrophages, monocytes, dendritic cells, and tissue epithelial cells as an acute response to injury and an initiator of the inflammatory process. PGE-2 is involved in several aspects of the wound healing cascade and is produced by inflammatory cells, epithelial cells, and fibroblasts. PGE-2 is a key inflammatory mediator, but it is also involved in later processes of wound healing, including inhibition of fibroblast migration,
stimulation of angiogenesis, and wound contraction.\textsuperscript{21–24} Furthermore, IL-1β and PGE-2 expression appears to be interrelated. IL-1β is responsible for the induction of gene expression for several key mediators associated with PGE-2 production. In fact, many biological activities associated with IL-1 are actually caused by increased levels of PGE-2 and vice versa.\textsuperscript{16}

The analysis of cytokines to document the severity of mucosal injury is not novel. As early as 1976, investigators found that concentrations of various constituents in gingival crevicular fluid (GCF) could be used to detect inflammation associated with active periodontal destruction.\textsuperscript{25} The authors reported that such assays should be useful for both clinicians and patients with gingivitis. Patients could read their own values on a GCF meter to self-evaluate their personal periodontal condition and the effectiveness of their home care program.\textsuperscript{25} More recently, Faizuddin et al\textsuperscript{26} demonstrated the ability to differentiate among clinically healthy gingiva, gingivitis with no attachment loss, and gingivitis with attachment loss, based solely on IL-1β concentration in GCF.

The current study seeks to use similar methods to document and quantify the inflammatory process in the vocal folds. The experimental questions are (1) can biochemical markers associated with wound healing be detected in vocal fold secretions in a rabbit vocal fold injury model? (2) If so, is there a temporal pattern associated with marker expression? (3) If a temporal pattern of expression is noted, is it consistent with the progression of events known to be associated with wound healing in other systems? Affirmative answers to these questions might provide insight into the acute wound healing process in the vocal folds, and it would suggest utility in biochemical markers collected from the vocal fold surface as a noninvasive means to detect and quantify some aspects of the acute wound healing process. However, the current study does not attempt to isolate the particular cell source of the markers. At least initially, determining the specific source(s) of the markers is not necessary to gauge the inflammatory process, which involves numerous cell types capable of producing the markers of interest. The current study seeks to describe the overall inflammatory milieu of the mucosal tissue, taking into account multiple cellular sources of such markers.

**METHODS**

The study was approved by the University of Pittsburgh Institutional Animal Care and Use Committee (IACUC) as a component of a broad investigation into the mechanics of acute wound healing in the larynx. Eighteen female White New Zealand rabbits were used. While the animals were under general anesthesia, secretions were collected from the superior and medial surface of the vocal folds with small pieces of Gelfoam sheeting (Upjohn Company, Kalamazoo, MI) held by forceps, using endoscopic guidance during direct laryngoscopy. The Gelfoam swabs containing the secretions were then placed in microfuge tubes and stored at \(-80^\circ\text{C}\) until analysis.

After collection of secretions, a unilateral vocal fold injury was induced by surgical removal of the mucosa down to the ligament upon gross examination. Three animals were then killed at each of six timepoints after the induction of the injury: 1, 5, 7, 10, 14, and 21 days. The larynx was removed for future histological analyses and secretions were immediately collected from the surface of the injured vocal fold using the technique described previously. To extract the secretions from the Gelfoam swabs, 500 mL of sterile saline solution was added to the microfuge tubes. The tubes were then mixed by vortex for approximately 5 seconds and centrifuged at 10,000 rpm for 3 minutes. The supernatant solution was collected in 1.5-mL microfuge tubes and labeled accordingly. This procedure was repeated with another 500 mL of saline solution, and the supernatant solution was collected in the same 1.5-mL microfuge tubes as in the previous wash. At that point, the microfuge tubes contained approximately 1000 mL. The volume of sample required for analysis was aliquoted. The remaining sample was placed in a microfuge tube and stored at \(-80^\circ\text{C}\) for later analysis. The entire procedure was performed cold to minimize the risk of denaturation, as occurs at room temperature and with repeated freezing and thawing. IL-1β and PGE2 were assayed by using ELISA kits according to the supplier’s recommended protocols (R&D Systems, Minneapolis, MN).

Marker concentrations were standardized based on secretion weight, because secretion volume could not be accurately measured. Protein as a baseline
measure was also problematic. Previously, our laboratory reported on the potential utility of the highly sensitive bicinchoninic acid (BCA) protein assay (Sigma Chemical Co., St. Louis, MO) as a means to standardize marker concentrations. However, it appears that such analyses may be susceptible to increased error when even a trace amount of blood is present in the sample. Microfuge tubes were weighed before secretion extraction. After extraction, the tube with Gelfoam swab was placed in a vacuum desiccation chamber for 5 days. The tube with Gelfoam swab was weighed again after desiccation, and the weight of the difference was used as the weight of the secretion. Concentrations for each biochemical marker were then divided by the weight of the secretion. Therefore, all results are reported in terms of picogram per gram of secretion.

**STATISTICAL ANALYSIS**

Two one-way between-subjects analyses of variance (ANOVA) were performed. For each analysis, marker concentration (pg/g secretion of IL-1β or PGE-2) was the dependent variable, and time after injury (days) was the independent variable. Posthoc comparisons were performed with the Tukey method to determine the specific differences in marker concentrations associated with the five timepoints after injury. The investigation-wide alpha level was set at 0.10 because of its exploratory nature. The alpha level was then adjusted to account for multiple statistical tests (α = 0.10/2). Therefore, the alpha level was set at 0.05 for each of the ANOVAs. An alpha of 0.05 was also used for the posthoc comparisons.

**RESULTS**

Detectable levels of both IL-1β and PGE-2 were obtained for all animals. Results are presented separately for each biochemical marker.

**IL-1β**

The main effect for IL-1β was significant (P = 0.001). Results are displayed in Figure 1. That figure illustrates that IL-1β concentration increased markedly in the acute phase after injury. Maximal expression occurred 1 day postinjury. Statistically, concentrations of IL-1β were significantly different from preinjury levels for every timepoint through day 5 (1 day, P = 0.003; 5 days, P = 0.011). Beyond 5 days after injury, statistically significant differences relative to preinjury levels were not obtained (7 days, P = 0.552; 10 days, P = 0.905; 14 days, P = 0.235, and 21 days, P = 1.00). Although numerically the concentration appeared to increase somewhat at day 14, that observation was not reliable because of a large standard error. It appears that IL-1β concentration is elevated early and begins to return to baseline level by 7 days after injury.

**PGE-2**

The main effect for PGE-2 was also significant in the ANOVA (P < 0.001). Results are displayed in Figure 2. Similar to IL-1β, PGE-2 expression appeared to increase 1 day after injury; however, that increase was not statistically significant (P = 0.150). Similar results were obtained 10 days after injury. Posthoc comparisons revealed insignificant findings for that timepoint (P = 0.234). The data appear to indicate increased PGE-2 concentrations at day 10. The concentration of PGE-2 at all other timepoints was significantly higher than at preinjury levels (5 days, P = 0.015; 7 days, P < 0.001; 14 days, P = 0.041; and 21 days, P = 0.005). Maximal expression occurred 7 days after injury. PGE-2 levels did not return to preinjury concentrations by 21 days, the endpoint of the current study. Thus, elevated levels of PGE-2 extended into the later phases of healing.

**DISCUSSION**

The primary experimental question was to determine whether biochemical markers associated with wound healing could be detected from secretions in a rabbit model of acute vocal fold injury. If detected, the next experimental question was to determine whether there was differential temporal expression of such markers, and, if so, whether expression was consistent with previous studies of the events associated with wound healing in other systems. Detectable levels of IL-1β and PGE-2 were obtained by using the rabbit model. That result strengthens conclusions from previous studies indicating the utility of the assay of vocal fold secretions to monitor and describe wound healing in the vocal folds.
Relative to the second experimental question, the data indicated a distinct pattern of expression for each of the two markers as a function of time after injury. Both mediators displayed increased concentrations in vocal fold secretions in the early post-injury period. Levels of IL-1β returned to baseline levels by 7 days after injury. In contrast, PGE-2 concentrations remained elevated through day 21 with maximum expression 7 days after injury. However, statistically significant differences were not achieved between preinjury levels and levels taken 1 day and 10 days after injury. The data suggest a trend of increased PGE-2 expression at those time-points. PGE-2 elevation at day 21 (the study endpoint) may indicate a role for this mediator in the later phases of the wound healing process.

Of relevance, a moderate degree of variability in the data was observed. Such variability may be related to several factors. This variability may reflect both within- and across-species disparities in response to injury. Animals may have responded differently to injury, altering the concentrations of biochemical markers associated with wound healing. Indeed, individual differences in the response to injury have been identified in studies of other tissues.26 Our laboratory has shown that vocal loading dramatically alters the profile of biochemical markers in mucosal secretions.15 The phonatory patterns of the animals were not controlled after injury; this may also contribute to variability in the data.

Relative to the third experimental question, the present data displayed a distinct pattern of expression that corresponds to the established roles of the two markers in the wound healing process. IL-1β is a key inflammatory mediator and a key initiator of the wound healing cascade after acute injury. In the current study, IL-1β expression was maximal in the acute phase (1 day) after injury, with relatively little expression at later time points (7–21 days). In contrast, the data indicated that PGE-2 has a role in both the inflammatory phase and later stages of wound healing in the vocal folds. Increased levels of PGE-2 were noted throughout the 21-day experiment with maximal concentrations 7 days after injury. Increased PGE-2 concentrations at later time points may be related to the inhibition of fibroblast migration or the initiation of wound contraction as previously suggested in both the skin and airway.21,22 However, this hypothesis is purely conjecture at this point.

To date, no study has attempted to correlate concentrations of biochemical markers in secretions collected from the vocal folds with concentrations of

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**FIGURE 1.** Results for IL-1β across time. Error bars represent standard error of the mean (SEM).
FIGURE 2. Results for PGE-2 across time. Error bars represent standard error of the mean (SEM).

the same biochemical markers within the tissue. This is a potential weakness of the current study that will be addressed in future reports. However, our laboratory has found that concentrations of key inflammatory mediators in secretions are significantly greater when collected from the site of injury versus uninjured vocal folds, as well as other control sites along the upper aerodigestive tract. These findings enhance the validity of the current investigation. Clearly, concentrations of wound healing markers in mucosal secretions capture some aspect of the wound healing response. Furthermore, the profile of biochemical markers in vocal fold secretions appears to correlate well with the expected temporal expression within the tissue, thereby providing encouraging data to stimulate further study.

Although further investigation of the present issues is certainly warranted, the current study sought to build on a series of studies suggesting the potential investigational use of ELISAs to document the wound healing process in the vocal folds. Results from the present study suggest that biochemical markers associated with wound healing can be detected in vocal fold secretions in a rabbit model of acute vocal fold injury. Furthermore, a temporal pattern of biochemical marker expression was found; this was consistent with previous studies regarding the specific role of both IL-1β and PGE-2 in the wound healing cascade in other systems.

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REFERENCES