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Structural and functional vocal fold epithelial integrity following injury

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Abstract

Objective—An intact epithelium is an important part of vocal fold defense. Damage to the epithelium can compromise vocal fold homeostasis and protection of the host tissue from viral and bacterial invasion. Elucidating the effects of damage on epithelial architectural and barrier integrity provides insight into the role of epithelium in protecting vocal folds. Using an animal model, we evaluated the time course of structural and functional epithelial restoration following injury.

Study design—Prospective, controlled animal study.

Methods—Forty rats underwent surgery to remove vocal fold mucosa unilaterally. Larynges were harvested at five time intervals between 3 to 90 days post injury and were prepared for histological and permeability analyses.

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Conflict of interest: None

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Levels of evidence: NA

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Results—Rapid restoration of structural integrity was demonstrated by return of a multilayerd epithelium, intercellular junctions, and basement membrane at five days post-injury. Atypical epithelial permeability was observed up to five weeks post injury.

Conclusion—Restoration of epithelial barrier integrity lags epithelial structural restoration. Consequently, epithelial regeneration cannot be equated with return of functional barrier integrity. Rather, ongoing leakiness of regenerated epithelium indicates that vocal folds remain at risk for damage, pathogen invasion and remodeling post injury.

Keywords

vocal fold; surgery; epithelium; basement membrane; permeability

Introduction

A stratified, squamous epithelium provides vocal folds with a protective barrier against mechanical, chemical, and biological insults. When the structural integrity of the epithelium is compromised, the vocal folds are vulnerable to damage and disease. Structural changes to the epithelium are implicated in the early pathogenesis of common vocal fold disorders such as vocal nodules[1,2], polyps[1], Reinke's edema[3] and granuloma[4–6]. Therefore, understanding how epithelium repair occurs following damage is important for optimizing prevention and treatment of many voice disorders. In the present study, we used an animal surgical model to create a temporal and spatial time line of epithelial recovery following injury. Our goal was to examine the relationship between the restoration of structural components of epithelium, cell-cell and cell-substrate junctions, and the return of functional integrity in epithelium to elucidate the role of epithelium in vocal fold defense following damage.

Vocal fold surgery in an animal model provides a controlled, clinically-relevant environment for examining healing following damage. Scarring is a relatively frequent, intractable consequence of surgery that is purported to negatively affect vocal outcomes for over a third of patients who undergo surgery[7]. While it is well-accepted that scarring alters the biomechanical properties of vocal fold lamina resulting in compromised vocal quality[8] and efficiency[9], the manner by which epithelial structural and functional integrity occurs and impacts the morphology and biological functions of vocal fold mucosa is not known. By identifying the timing and extent of restoration of the structural and functional integrity of epithelium, we can examine the impact of injury on epithelial defense.

Vocal fold defense is maintained in part by epithelium and basement membrane[10] (Erickson et al., In Review). They provide the vocal folds with an important physical and biochemical barrier against mechanical, chemical and pathogen insults. Epithelial cells are connected by intercellular junctions which give epithelium structural and functional integrity. These junctions include tight junctions[11,12], gap junctions (e.g. connexin 43, a marker of a predominant type of gap junctions)[13], adherens junctions [14], and desmosomes. They hold epithelial cells together during vibratory stress, provide a selective barrier to paracellular diffusion, and allow intercellular communication. The epithelial cells adhere to the basement membrane via hemidesmosomes. Secure attachment to the basement membrane is necessary to adhere the epithelium to the underlying lamina propria as well as provide an additional

selective barrier for the passage of materials to and from the vocal fold surface. In this study, we examined the extent to which epithelium, basement membrane and each of these junctions were present during the early and late stages of healing following injury as adequate vocal fold defense requires presence of each of these components.

The timing and extent of restoration of epithelial barrier function following epithelial injury is not yet known. Furthermore, the relationship between regeneration of cell-cell and cell-substrate junctions and return of functional integrity in epithelium has not been explored. We anticipated that restoration of functional integrity would lag behind return of structural integrity. Branski and colleagues[15] observed a confluent, multilayered epithelium two weeks following injury, however, they reported that poor dermal-epidermal adhesion was present at three weeks following surgery. Thus, we surmised that return of a stratified epithelium cannot be equated with return of epithelial barrier integrity. In the present study, we sought to assess epithelial permeability during the immediate and later phases of wound healing in a rat model using an established method[16,17]. Concurrently, we examined the time line for regeneration of structural features of the epithelium and basement membrane to shed light on the relationship between architectural and barrier integrity. We hypothesized that chronic leakiness would arise following vocal fold injury and that restoration of epithelial structure would be a necessary, but not sufficient, prerequisite for return of barrier function. We expected to observe increased epithelial permeability post-surgery despite regeneration of a confluent, stratified epithelium.

Materials and methods

Animals

Forty two- to four-month-old Sprague Dawley rats were used in this study. The animal protocol was implemented with prior approval from the Institutional Animal Care and Use Committee of the University of Wisconsin-Madison. All animals underwent unilateral vocal fold surgery. Briefly, rats were anesthetized by exposure to isofluorane (2% to 3% delivered at 0.8–1.5 L/minute) and intraperitoneal injection of 90 mg/kg of ketamine hydrochloride and 9 mg/kg of xylazine hydrochloride and placed in a near-vertical positions on a custom designed stand. Salivation was controlled through intraperitoneal injection of atropine sulfate (0.05mg /kg). The mouth was secured in an open position using a custom-made device[18]. The vocal folds were visualized with a 1.9mm 30-degree endoscope (Karl Storz Endovision, Inc., Charlton, MA). Using a 25-gauge spinal needle, the epithelium and lamina propria of one vocal fold was removed to expose the underlying thyroarytenoid muscle. Eight animals were euthanized and their larynges were harvested at each of five time points (3, 5, 14, 35 and 90 days) post-surgery (N=40). Three larynges were processed for routine histology and immunohistochemistry and five were processed for transmission electron microscopy at each time point. A pathologist (DTY) confirmed presence of vocal fold injury in tissue sections obtained from each animal.

Histology and immunohistochemistry

Three larynges per time point (n=15) were fixed in 10% neutral phosphate-buffered formalin (pH of 7.0) overnight at room temperature, processed, and embedded in paraffin. Serial

coronal sections, 5μm thick, were cut along the entire length of the membranous vocal folds. Routine hematoxylin and eosin (H&E) staining was performed on sections at 100μm increments to identify injured regions of the vocal fold.

Standard IHC was performed using primary antibodies against an adherens junction marker e-cadherin (1:500) and a basement membrane marker, laminin 5 (1:50). Both antibodies were purchased from Abcam (Cambridge, MA). Antigen retrieval using sodium citrate and heat was performed and antibodies were applied overnight at 4 °C. Labeling was detected using horseradish peroxidase and visualized with diaminobenzidine (DAB) as the chromagen prior to counter staining with hematoxylin and mounting. Sections were viewed with a Nikon E600 microscope (Nikon, Melville, NY) and photographed using an Olympus DP71 microscope digital camera (Tokyo, Japan). Each section contained an injured and uninjured vocal fold. The uninjured vocal fold from each larynx served as a within animal control.

Transmission electron microscopy

For standard transmission electron microscopy, two larynges per time point (n=10) were cut into five 200μm coronal sections with a vibratome and were immersion fixed in 2.5% glutaraldehyde and 2% paraformaldehyde in a 0.1M sodium cacodylate buffer (pH 7.4) overnight at 4 °C. The samples were processed using routine techniques. Briefly, sections were washed in 0.1M sodium cacodylate buffer and postfixed in 1% osmium tetroxide in the same buffer for two hours at room temperature. Samples were dehydrated in graded ethanol series, rinsed twice in propylene oxide and embedded in Epon 812 epoxy resin (Polysciences Inc., Warrington, PA) under vacuum. Finally, the samples were flat embedded between glass slides. Each section contained an injured and uninjured vocal fold. The latter served as a within animal control.

After resin polymerization, one of the two glass slides was removed and blank resin cylinders were glued to the tissue samples. The samples were thin sectioned for TEM using a Leica EM UC6 Ultramicrotome and stained with Reynolds lead citrate and 8% uranyl acetate in 50% EtOH to increase contrast. The thin sections were viewed with a Philips CM120 electron microscope and images were captured with a MegaView III side-mounted digital camera.

For permeability analysis, the above standard transmission electron microscopy protocol was used. However, following coronal sectioning of three larynges per time point (n=15) into thick (200μm) sections, a heavy metal tracer, lanthanum nitrate (final concentration 1%; Electron Microscopy Sciences, Hatfield, PA) was added to the fixative and to reagents at each subsequent stage of processing including buffer washes, osmium tetroxide and alcohol rinses. The tissue samples were thin-sectioned and viewed as described above.

Results

Structural analysis

Electron microscopy revealed return of a multilayered epithelium within three days after injury. Tight junctions, gap junctions, adherence junctions, and desmosomes were observed

at all time points after injury (Figure 1). Restoration of adherens junctions was confirmed by immunohistochemistry. Positive staining for e-cadherin, a marker of adherens junctions, was observed in all animals at each time point post-injury beginning at day 3 and in an uninjured vocal fold (Figure 2).

Permeability analysis

Despite rapid restoration of epithelial structural integrity, the epithelium demonstrated excessive permeability for at least two weeks post-injury in all larynges. Permeation of lanthanum nitrate was observed through all epithelial layers including the basal cell layers in all animals at 3, 5 and 14 days post-injury (Figure 3). Lanthanum nitrate was also observed in one of three larynges harvested at 35 days post-injury. In contrast, permeation of the tracer did not occur at 90 days post injury or in an uninjured tissue. Rather, lanthanum nitrate was observed to coat the superficial layer of cells exclusively.

Basement membrane

Lamina lucida, lamina densa and hemidesmosomes were not observed at 3, 5 and 14 days post-injury but visible at 35 and 90 days post injury. Hemidesmosomes were present at both time points (Figure 4). Immunohistochemical findings were consistent with these observations; laminin 5, a component of basement membrane was absent at day 3 but present by day 5 in all vocal folds, as well as in an uninjured vocal fold (Figure 5).

Discussion

An intact epithelium is an important component of vocal fold defense. Damage to the epithelium can compromise vocal fold homeostasis and render host tissue vulnerable to viral and bacterial infection. Using an animal model, we evaluated the time course of structural and functional epithelial restoration following injury following resection of the epithelium and lamina propria. Here, we examined the pattern of healing of epithelium following an injury of a similar extent and depth. Rapid restoration of epithelial barrier function would suggest that vocal folds are well-protected from the external environment during healing of the lamina propria. Conversely, delayed return of epithelial integrity would indicate that the vocal folds are vulnerable to damage, irritation or infection after injury. Further, continued epithelial leakiness would be consistent with a role for compromised barrier integrity in chronic mucosal remodeling following surgery. Elucidating the timeline for return of epithelial defense would permit appreciation of the potential roles of epithelium and basement membrane in modulating healing thus providing a more complete understanding of wound healing and, consequently, an opportunity to develop interventions to protect vocal folds and hasten restoration of a protective barrier

In the present study, we evaluated the time course of both structural and functional epithelial restoration following injury. We observed rapid regeneration of epithelium. A multilayered, confluent epithelium and intercellular junctions returned within three days post-injury. The basement membrane was absent for at least two weeks post-injury. At five weeks post-injury, a complete basement membrane was observed. These findings are consistent with the timeline for regeneration of intercellular junctions and basement membrane reported

elsewhere. For example, e-cadherin, a key component of adherens junctions, was seen in rats at 3 days post-injury[19]. Components of the basement membrane were observed to return one to two weeks post-injury in dogs. For instance, Cho and colleagues[20] observed emergence of laminin 5 by one week post-injury with normal staining pattern at 2 weeks post-injury in a dog model. They also reported that normal basement membrane ultrastructure, including presence of hemidesmosomes and lamina lucida and densa, was fully restored by 4 weeks post-injury under conditions of vocal rest.

Despite the rapid epithelial regeneration, the epithelium remained leaky for at least two weeks post-injury. One of three vocal folds was observed to be leaky at five weeks post-injury. Our findings suggest that epithelial integrity is compromised during the early phase of wound healing and indicate that regeneration of an epithelium cannot be equated with restoration of epithelial defense. Further, regeneration of intercellular junctions is not a sensitive indicator of epithelial healing and restoration of barrier function. We surmise that presence of a multilayered confluent epithelium, a continuous basement membrane, and intercellular junctions is likely a necessary, but not sufficient, condition for restoration of epithelial functional integrity.

Epithelial barrier function can be examined using permeability assays or by measuring transepithelial resistance (TER). We elected to use the former approach for two reasons. First, by measuring permeability using a lanthanum nitrate assay, we were able to confirm that tight junctions were not functional post-injury. Had we observed low transepithelial resistance, we could have speculated that the tight junctions were disrupted given that tight junctions are a key regulator of permeability. However, we could not have ruled out the possibility that other factors or artifacts unrelated to the integrity of tight junctions, such areas of absent epithelium, could have caused a low resistance measure. Second, the two methods are complementary. An increase in vocal fold epithelial permeability typically is associated with a fall in transepithelial resistance. Zhang and Fisher[21] reported that tight junctions play an important functional role in vocal fold epithelium. They demonstrated that when tight junctions are disrupted by histamine, transepithelial resistance falls and epithelium becomes permeable to horseradish peroxidase. Like lanthanum nitrate, horseradish peroxidase is a protein tracer used to assess paracellular permeability.

We are mindful of several limitations to the present study. First, rats offer a well-established model of vocal fold injury. However, while the sequence of healing mirrors that reported in larger animals and humans, their shortened lifespan results in an accelerated rate of healing. Thus, one must be cautious when extrapolating findings to clinical practice. Second, the focus of this study was on the contribution of epithelial architecture on barrier function. We did not assess the role of chemical and immunological defense on epithelial barrier function. It is possible that defense responses to injury such as IgA, antimicrobial, lysosome, or mucin secretion may have occurred in and mitigated the potentially damaging effects of increased epithelial permeability on vocal fold health. Future studies are warranted to address these other facets of epithelial defense. Finally, we cannot extrapolate our findings to the effects of increased permeability during wound healing on vocal fold vulnerability to common challenges in clinical populations. We can speculate, however, that bacterial translocation may occur due to permeability changes based on findings in epithelia throughout the body.

For example, *pseudomonas aeruginosa* internalization occurs in airway epithelia when intercellular junctions are disrupted[22]. Presence of this bacteria during wound healing is problematic as it delays epithelial healing in the airway[23]. Loss of intercellular junction integrity can also permit penetration of viruses to the basal layer of cells or to the host tissue. For example, studies have shown that disruptions to epithelial integrity renders host tissue vulnerable to viral infections such as the human immunodeficiency virus (HIV)[24] and the human papillomavirus (HPV)[25]. Additionally, Zhang and Fisher[21] have demonstrated that tight junction integrity is important for vocal fold health as tight junctions maintain electrophysiological homeostasis in ovine vocal fold epithelium. The authors noted that tight junction integrity is critical for normal transport of water and solutes across the epithelium. They further speculated that intact tight junctions are important for blocking luminal pathogens from reaching the underlying vocal folds.

Reflux presents an additional common challenge to patients. Erickson and Sivasankar[26] reported a decrease in transepithelial resistance following exposure of excised vocal folds to acid and pepsin. Clues to the mechanism that may drive this decrease in barrier function were identified by Koufman and colleagues[27]. The group showed previously that e-cadheren, a marker or adherens junctions, was partially or fully absent in about one half of laryngeal specimens with laryngopharyngeal reflux (LPR). However, the group noted previously that it was not known whether this was a cause or consequence of laryngeal inflammation in specimen with LPR[28]. Elsewhere Roh and Yoon[29] reported that exposure of injured vocal folds to simulated reflux (acid and pepsin), resulted in an increased incidence of granulation formation and a greater inflammatory response, relative to unexposed injured vocal folds. While we did not study the effects of acid and pepsin on wound healing, based on these studies, we can speculate that disruption to epithelial intercellular junctions and increased epithelial permeability may render the underlying tissue at greater risk for damage compared to an intact epithelium.

Conclusion

We have shown that return of a stratified epithelium cannot be equated with return of epithelial barrier integrity. Therefore, histological assessment of epithelium and basement membrane is not sufficient for evaluating vocal fold defense. Rather, functional measures, such as the permeability assay used in the present study are necessary to evaluate vocal fold defense. In the present study, restoration of epithelial functional integrity lagged regeneration of a structurally intact vocal fold mucosa. Further examination of the functional integrity of epithelium and basement after injury is warranted to better appreciate their potential role in modulating mucosal remodeling post injury results permitting the development of interventions to protect vocal folds and hasten restoration of a protective barrier.

Acknowledgments

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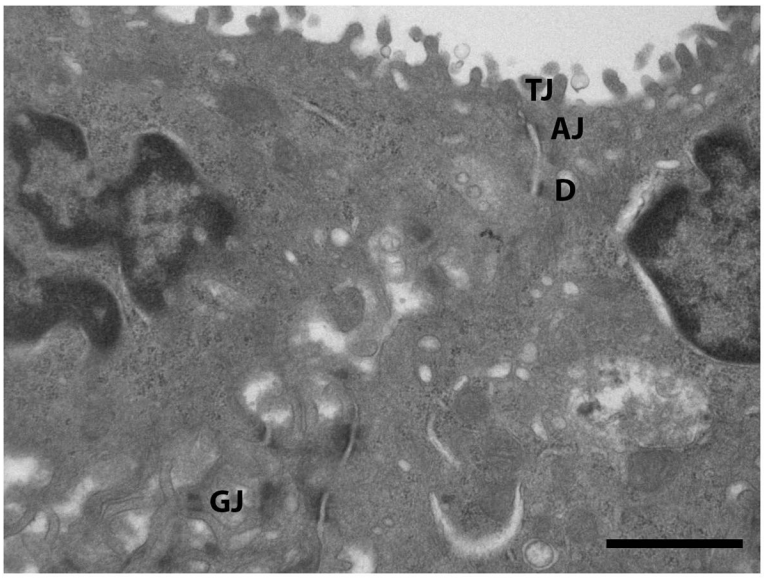

Figure 1.
Intercellular junctions were observed via transmission electron microscopy to be present by 3 days post-injury including tight junctions (TJ), adherens junctions (AJ), gap junctions (GJ), and desmosomes (D) (scale bar: 1 micron)

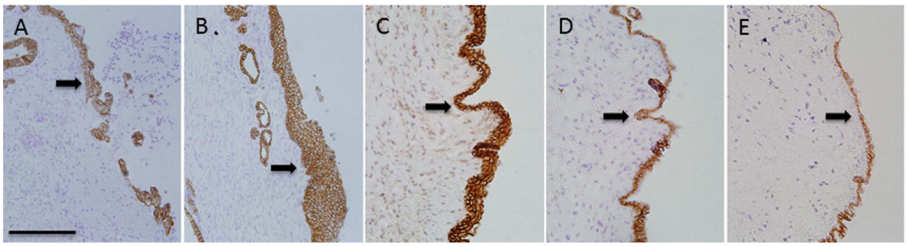

Figure 2.

Adherens junctions (arrows) were observed in epithelium adjacent to the denuded wound site 3 days post injury (A) using immunohistochemistry. Staining was present in regenerated epithelium at 5 (B), 14 (C) and 35 (D) days post-injury and in uninjured epithelium (E) (scale bar: 200 microns).

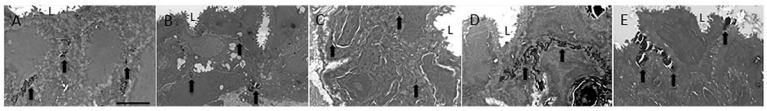

Figure 3.

Lanthanum nitrate (arrows) permeated epithelial layers at 3 (A), 5 (B), 14 (C), 35 (D) days post-injury as observed with transmission electron microscopy. No permeation was observed at 90 days post-injury (E; scale bar: 2 microns), instead lanthanum nitrate was confined to the luminal surface of the vocal folds (L)

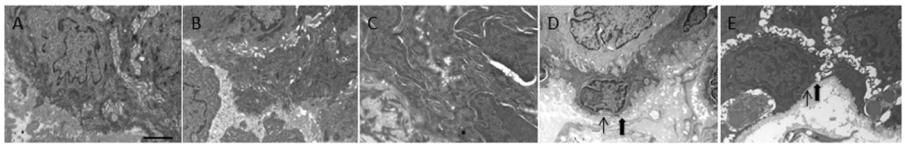

Figure 4.

Basement membrane was not observed at 3 (A), 5 (B), 14 (C) days post-injury using transmission electron microscopy. It was present at 35 (D) and 90 (E) days post injury. Lamina densa (thick arrow) and lamina lucida (thin arrow) were present in the regenerated basement membrane (scale bar: 2 microns).

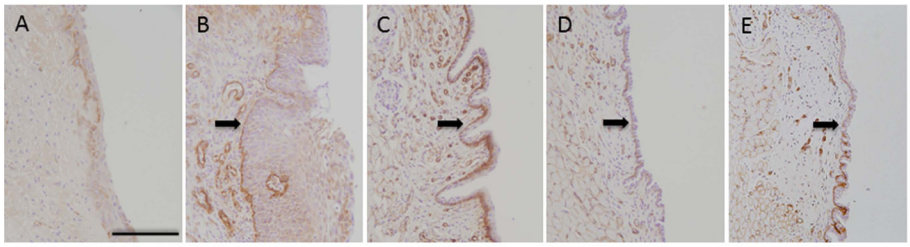

Figure 5.

Laminin 5 staining (arrows) was absent from the basement membrane region at 3 days post-injury (A). Staining was present at 5 (B), 14 (C) and 35 (D) days post-injury and in uninjured vocal folds (scale bar: 200 microns).