Vocal Fold Fibroblast Response to Mechanical Stress

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The role of exercise in vocal fold wound healing has been overlooked. Data from numerous other systems suggest a positive role of tissue mobilization to facilitate optimal wound healing and biomechanically superior tissue. The current study sought to investigate the potential role of mechanical signaling to attenuate the inflammatory and alter the synthetic properties of fibroblasts cultured from the vocal folds. Vocal fold fibroblasts were subjected to one of four conditions: no treatment, IL-1β alone, mechanical stress alone, or mechanical stress plus IL-1β. Results suggest that mechanical stress may limit the inflammatory phenotype of vocal fold fibroblasts in the short-term (4 hours), but not in the long-term (24 hours). In fact, 24 hours of mechanical stress may actually increase the inflammatory response. In addition, neither IL-1β nor mechanical stress had an effect on vocal fold fibroblast synthesis of extracellular matrix proteins. As a potential explanation for the current findings, it is hypothesized that the vocal folds may be more resilient to mechanical stress given the inherently stressful environment associated with phonation.

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PREFACE

I have been so lucky to be surrounded by incredible people throughout my doctoral studies. Without them, I surely would not have survived. I must first give thanks to Dr. Verdolini. She molded me into a better scientist, writer, and person. Regardless of how many times I have cursed her name, I am forever in debt to her. She is an extraordinary person and a true mentor. Most importantly, I consider her a life-long friend.

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Dr. Hebda took a chance on a student with no basic science background. She took a blank slate and developed a quasi-scientist. Although I may not have verbalized it, I am so appreciative of your mentorship and support over the past several years. I wish you the best of luck and look forward to future collaborations. And finally, Dr. Agarwal, thank you for making me feel at home in Columbus. Without your help, support, and Indian food I would have given up.

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INTRODUCTION

Voice disorders appear to be the most common communication disorder across the lifespan. Estimates indicate that anywhere from 3-9% of the population has some type of voice dysfunction at any given time (Verdolini & Ramig, 2001). Voice disorders include a broad spectrum of conditions, ranging from life-threatening carcinoma to mild laryngitis with minimal impact on communicative function. Regardless, the potential social and occupational ramifications associated with voice disorders are substantial. Focusing on teachers, who have the most common occupational label in the U.S. (about 5.6% of the employed population; 1999 National Occupational and Wage Estimates), and also have a high occurrence rate of voice disorders, the data are striking. Anywhere from 20-33% of *all* teachers miss work due to voice problems (Sapir, Keidar, & Mathers-Schmidt, 1993; Smith, Gray, Dove, Kirchner, & Heras, 1997). Furthermore, there is evidence suggesting voice problems in teachers may reduce cognitive functioning in students (Morton & Watson, 2001). Costs due to voice therapy, surgery, and substitute personnel are conservatively estimated at around $2 billion annually in the U.S., for teachers alone (Verdolini & Ramig, 2001). This figure is likely dramatically higher in the population at large.

It is likely that the source of most voice problems in teachers and other high-risk populations is phonotrauma. Phonotrauma, or the microinjury associated with phonation, is primarily due to perpendicular impact stress between the vocal folds (D. A. Berry et al., 2001; Jiang & Titze, 1994; Titze, 1994). High intrafold collision forces likely intensify tissue damage and the development of lesions due to increasing levels of mechanical stress (Titze, 1994). In addition, vertical shear stress associated with vocal fold oscillation may modify cellular behavior (Gunter, 2003).

Traditional models of voice therapy for phonotrauma emphasize voice conservation (Boone & McFarlane, 1994; Colton & Casper, 1996). In those models, a "vocal diet" approach is utilized, in which individuals are encouraged to minimize the quantity and/or loudness of phonation (Froeschels, 1943; Roy et al., 2001; Roy et al., 2002). Typical therapeutic gestures involve "quiet breathy", or "confidential" voice (Casper & Murry, 2000). Biomechanically, this approach is sensible; quiet-breathy phonation should minimize pathogenesis by reducing impact stress between the vocal folds (D. A. Berry, Verdolini, K., Montequin, D.W., Hess, M.M., Chan, R.W., Titze, I.R., 2001; Jiang & Titze, 1994; Titze, 1994). Moreover, *in vivo* data appear consistent with the traditional bias towards voice conservation following phonosurgery. Specifically, enhanced resolution of the basement membrane zone has been noted in an animal model of phonosurgery following recurrent laryngeal nerve section-induced voice rest, compared to resolution in animals encouraged to phonate (Cho, Kim, Lee, Kim, & Park, 2000). However, no data currently exist validating the role of voice rest or conservation following phonotraumatic injury in animals or humans. Poor patient compliance and the inherent social ramifications associated with voice rest further limit its therapeutic value.

Furthermore, recent thoughts have challenged the traditional approach of voice conservation (Verdolini, 2000). There is emerging evidence from research using other tissues suggesting a positive role for some forms of exercise, instead of rest, to enhance resolution of inflammation as well as to optimize tissue mechanics both *in vitro* and *in vivo*. With regard to voice, recent clinical observations have suggested that the use of large-amplitude, low-impact vocal fold oscillations associated with "resonant voice" (Verdolini, Druker, Palmer, & Samawi, 1998) may assist in the recovery of vocal fold inflammation associated with acute phonotrauma, and also in the optimization of short- and long-term functional voice use.

The cellular explanation for such observations in the vocal folds has not been elucidated. This lack of insight is particularly unfortunate because the goal of clinical management, in many cases, is enhanced healing of injured mucosa and re-establishment of functional tissue. Exercise that can be shown to yield improved tissue profiles at the molecular level should be included in the therapeutic milieu. To date, no such exercises have been formally examined in the literature. The current series of experiments sought to address this issue by examining the potential for vocal fold oscillation to enhance resolution of vocal fold inflammation and improve long-term extracellular matrix composition following injury. The studies utilized a well-established *in vitro* model of cyclic mechanical strain applied to vocal fold fibroblast monolayers to investigate a broad spectrum of wound healing events associated with inflammation and its sequelae. The first experiment examined the reduction in inflammatory response associated with cyclic tensile strain. The second experiment investigated the synthesis of extracellular matrix components in response to inflammation and cyclic tensile strain. This latter issue is pertinent not only to acute inflammation, but also to long-term extracellular matrix composition following injury.

The studies addressed the following specific aims:

Specific Aim 1: Examine the pro-inflammatory effects of interleukin-1β (IL-1β) on vocal fold fibroblasts (VFF), and quantify the effects of cyclic tensile strain (CTS) on those effects, by:

(a) Observing mRNA expression of proinflammatory molecules cyclooxygenase-II (COX-II) and inducible nitric oxide synthase (iNOS), as well as the synthesis of inflammatory mediators such as prostaglandin E-2 (PGE-2) and nitric oxide (NO) in vocal fold fibroblasts in response to IL-1β exposure, and by

(b) Observing proinflammatory gene induction (COX-II and iNOS), and pro-inflammatory mediator (PGE-2 and NO) synthesis in vocal fold fibroblasts exposed to IL-1β alone, CTS alone, both IL-1β and CTS, or no treatment.

Specific Aim 2: Examine the effects of interleukin-1β (IL-1β) on extracellular matrix (ECM) synthesis in vocal fold fibroblasts (VFF), and quantify the actions of CTS on ECM synthesis subsequent to IL-1β exposure, by:

(a) Observing the mRNA expression of extracellular matrix components (collagen, hyaluronic acid, fibronectin, and decorin) and synthesis of collagen and hyaluronic acid in vocal fold fibroblasts exposed to IL-1β, and by

(b) Observing extracellular matrix synthesis in vocal fold fibroblasts exposed to IL-1β alone, CTS alone, both IL-1β and CTS, or no treatment.

Rationale for the Current Series of Experiments

Wound healing, in general, is a highly coordinated sequence of events involving complex interactions among cells, extracellular matrix, cytokines, and other mediators. Wound healing is classically divided into three phases: inflammation, proliferation, and remodeling (Kirsner & Eaglstien, 1993). The primary focus of the current investigation is the inflammatory process. Inflammation is described most simply as the influx of blood-borne cells and the subsequent release of cytokines and mediators into the wound area. Among cytokines and mediators associated with inflammation, interleukin-1β (IL-1β) has been well described as a key pro-inflammatory mediator involved in the acute response to injury, and as an initiator of the inflammatory cascade (Dinarello, 1997, 2000). For example, IL-1β has been shown to play a significant role in cartilage destruction in chronic inflammatory diseases such as osteoarthritis

and rheumatoid arthritis (Bandara, Georgescu, Lin, & Evans, 1991; Flugge, Miller-Deist, & Petillo, 1999).

The acute inflammatory response in the vocal folds remains poorly described, forcing clinicians to make treatment decisions based on findings from other tissue types. Initial findings have indicated that, similar to other tissue, IL-1β is implicated in vocal fold inflammation associated with phonotrauma. Specifically, dramatic shifts in IL-1β concentration have been noted as early as 10 to 20 minutes following acute phonotrauma in a human subject (Verdolini, Rosen, Branski, & Hebda, 2003b). These data are shown in Table 1.

	IL-1 β (µg/ml of protein)
Baseline	10
10-min Post-Loading	84
20-min Post-Loading	123

Table 1. IL-1β concentrations in response to vocal loading.

Secretions were collected from the surface of the vocal fold folds before and after vocal loading. Data from Verdolini, Rosen, Branski, and Hebda, 2003a.

A relevant question regards how the subsequent inflammatory cascade can be studied in detail. If the goal is to study the effects of mechanical perturbation on wounded tissue, as in the present studies, an *in vitro* model is the most straightforward experimental option. The process involves exposing cultured cells to IL-1β, thus triggering an inflammatory phenotype. The inflammatory response of cultured cells can then be assessed via both gene expression methods as well as by methods examining cell secretion of inflammatory mediators.

Inflammatory markers such as inducible nitric oxide synthase (iNOS) and its product nitric oxide (NO) or cyclooxygenase-II (COX-II) and one of its products prostaglandin E2 (PGE-2) have been described extensively in the wound healing literature, providing rationale for the current investigation. PGE-2 is a pro-inflammatory molecule synthesized by cyclooxygenase-II (COX-II). Elevated levels of PGE-2 in vocal fold secretions from patients with established vocal fold lesions have been described. These data are shown in Figure 1. Relevant to the present study, quantification of both COX-II gene expression and PGE-2 synthesis *in vitro* provides insight into the vocal fold fibroblast response to injury, and potentially into the effect of mechanical stress on the inflammatory process in the vocal folds.

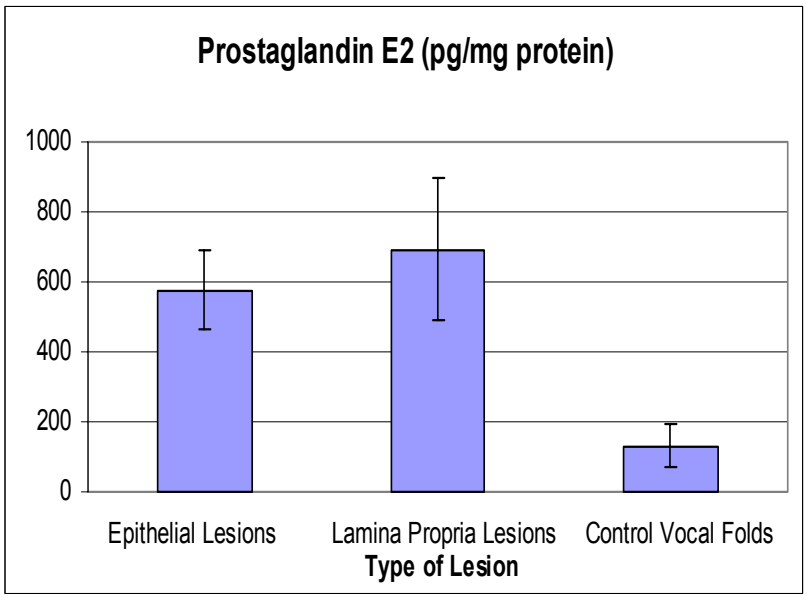

Figure 1. PGE-2 Concentrations in patients with vocal fold lesions.

Secretions were collected from the surface of the vocal folds in both patients with various vocal fold lesions and patients with clinically normal vocal folds (Branski, Verdolini, Rosen, & Hebda, 2004). Copyright permission courtesy of Annals Publishing Group.

In contrast, the role of iNOS and NO in vocal fold inflammation has not been characterized. NO is produced via inducible nitric oxide synthase (iNOS) as a response to

cytokine activation (IL-1β) following injury or bacterial invasion (Nathan, 1992; Nathan & Xie, 1994). The current study seeks to determine the potential role of NO (and iNOS) in vocal fold inflammation. Numerous studies have suggested a positive role for NO in the wound healing response in other tissues. However, NO is also implicated in tissue destruction in a variety of rheumatic diseases (Abramson, Amin, Clancy, & Attur, 2001).

Beyond studying the acute inflammatory response, the current work sought to determine the potential long-term ramifications of vocal fold inflammation with regard to ECM production. The synthetic phenotype of numerous other cell types has been modulated via patent mechanical signaling pathways. The current study seeks to determine if vocal fold fibroblasts respond similarly, thereby suggesting that therapeutic tasks in the acute stage following injury may alter the long-term extracellular profile of the vocal fold architecture. Of particular interest for the current proposal are collagen, hyaluronic acid, fibronectin, and decorin because of their relevance for wound healing and scar formation in the vocal folds.

Based on data from other tissue, it was hypothesized that inflammation would reduce proteoglycan synthesis. Therefore, the main hypothesis of the current investigation was that mechanical stress would attenuate inflammation-induced reduction in proteoglycan synthesis. Proteoglycans, a class of interstitial macromolecules found in the vocal folds, have been implicated in vocal fold scar formation (Pawlak, Hammond, Hammond, & Gray, 1996; Thibeault, Bless, & Gray, in press). Proteoglycans consist of a protein core with a linkage protein that attaches to the cores of other proteins, carbohydrates, and/or lipids (Ruoslahti, 1988). Three subgroups of proteoglycans have been identified in the vocal folds: (1) small, interstitial matrix proteoglycans such as decorin, biglycan, and fibromodulin, (2) large, aggregating chondroitin sulfate proteoglycans including aggrecan and versican, and (3) heparin sulfate

proteoglycans (Pawlak et al., 1996). Proteoglycans are especially important because they appear to be critical in the regulation of other matrix proteins in the vocal folds, primarily collagen (S. D. Gray, Titze, Chan, & al, 1999). Of particular interest is the small, interstitial proteoglycan, decorin, which is found primarily in the superficial layer of the lamina propria. Decorin is thought to bind collagen, delay fibril formation, and induce the formation of thinner fibrils (Hardingham & Fosang, 1992). Decreased decorin has been found in vocal fold scar (Thibeault et al., in press). Therefore, the inflammatory response with regard to proteoglycan production and specifically, decorin may provide insight into long-term functional outcomes associated with vocal fold injury and inflammation subsequent to exercise.

1. Further Background

The previous pages provided a basic foundation and rationale for the current experiments. The next sections provide comprehensive background information. The experiments were rooted in both the basic wound healing literature as well as recent findings describing the cellular response to cyclic tensile strain in the context of inflammation. Both of these issues warrant in-depth discussion to justify the current project. The following pages provide a foundational background in (1) the generalities of wound healing, including wound healing of the airway; (2) the role of "exercise" in the wound healing cascade; and (3) the cellular and non-cellular anatomy of the vocal folds.

1.1. Wound Healing

When injury disrupts the normal architecture of an organ, a fibroproliferative response commences resulting in fibrosis. Disruption in the normal healing process can produce even less desirable outcomes. For example, prolonged inflammation can yield more extensive tissue damage in conditions such as osteoarthritis. In addition, disease processes such as diabetes,

Cushing's syndrome, malnutrition, and sepsis disrupt the normal healing process and can lead to non-healing wounds or excessive fibrosis (Clark, 1998).

Wound healing is typically divided into distinct phases. However, these phases are actually neither linear nor clearly differentiated. Wound healing is a dynamic, interactive process involving blood elements, cells and extracellular matrix. Simply,uninterrupted wound healing consists of three temporally-concatenated phases: inflammation, tissue formation, and tissue remodeling (Clark, 1998). Relevant to the current project, particular detail is dedicated here to the review of inflammation as well as mesenchymal cell behavior involved in the formation of novel tissue at the site of the injury. Mesenchymal cells, primarily fibroblasts, are responsible for not only homeostasis of the extracellular matrix, but also for the production of new ECM following injury. In the vocal folds, this process is imperfect, yielding fibrosis and biomechanical limitations.

The basic goal of wound healing is the resolution of epithelial defects and reconstitution of functional tissue. Generally, clinical management of wound healing is concerned with non-healing, chronic wounds. Non-healing wounds do not appear to be a relevant entity in the vocal folds. To the contrary, problematic wound healing in the vocal folds involves an exaggerated or exuberant response, producing vocal fold fibrosis and dysphonia. Stated differently, in the vocal folds, the concern in wound healing is the formation of excessive neo-matrix within the lamina propria. Thus, although a review of wound healing in the skin can serve as a general framework for the present studies, generalization to the vocal folds is approached with caution.

Another caution regarding the present review is that the anatomic and physiological differences between the skin and vocal folds may limit the application of dermal wound healing principles to the vocal mechanism. Anatomically, the vocal folds have no dermal layer. Instead,

the epithelial lining of the vocal fold attaches to the underlying lamina propria via the basement membrane zone (BMZ). Epithelial-lamina propria re-attachment following injury is a critical component of the wound healing response in the vocal folds. Finally, the biophysical demands placed upon the vocal folds are unique. Continuous microtruama associated with phonation likely yields a series of wound healing events that are unique to the vocal folds. However, given that the vocal folds undergo marked mechanical stresses associated with modal phonation, there appears to be a threshold of injury that likely depends on the degree of trauma and/or the duration of trauma that stimulates a full-blown wound healing response.

Despite anatomic and physiologic discrepancies between skin and the vocal folds, a review of the generalities of dermal wound healing may serve as a framework for experimentation on the would healing process in the vocal folds. A concise review of the literature poses a challenge. Volumes could be dedicated to each phase of wound healing. Only cursory descriptions of the processes are included in the current review.

1.1.1. Inflammation

The series of biochemical events immediately following injury are collectively referred to as inflammation. This acute response ensures immune competence and orchestrates later wound healing events (Guirao & Lowry, 1996). The inflammatory process is triggered by damage to the cutaneous vasculature causing extravasation of blood constituents into the wound area (Trowbridge & Emling, 1997). In turn, extravasation of blood constituents stimulates inflammatory cells within the tissue to release chemotactic mediators, cytokines, and growth factors, which recruit neutrophils and monocytes to the injury site. In addition, these mediators initiate the repair phenotype in mesenchymal cells (Hackam & Ford, 2002).

One of the primitive goals of the inflammatory phase is to stop blood flow at the site of the wound. Such stoppage is called hemostasis or coagulation. Blood flow from damaged vessels is mechanically blocked by the formation of a fibrin clot or plug. Clot formation is the end-product of a myriad of processes collectively termed the "coagulation cascade." A critical component of the coagulation cascade is platelet aggregation at the wound site due to the presence of extraluminal collagen and tissue procoagulant factors from the injured cells (Kirsner & Eaglstein, 1993; Meyer, Frojmovic, & Vic, 1979). In addition, alpha granules released by aggregating platelets enhance clot formation by way of von Willebrand factor, fibrinogen, fibronectin, and thrombospondin (Clark, 2001; Plow, Loftus, Levin, & al, 1986; Terkeltaub & Ginsberg, 1988). Platelet aggregation results in the release of multiple cytokines including transforming growth factor-beta (TGF-β), epithelial growth factor (EGF), and platelet-derived growth factor (PDGF), which is mitogenic and chemotactic for fibroblasts (Katz, Alvarez, Kirsner, Eaglstein, & Falanga, 1991; Kirsner & Eaglstein, 1993). This process appears to be self-limiting and thus excessive clot formation is avoided. Primarily, prostacyclin produced by endothelial cells inhibits platelet aggregation and thereby limits the extent of aggregation at the site of the wound (Moncada, Gryglweski, Bunting, & Vance, 1976).

In addition to hemostasis, the coagulation cascade also facilitates mesenchymal cell migration into the wound primarily via platelet-derived fibrinogen converted to fibrin. Fibrin (catalyzed by the production of thrombin) is the main component of the provisional matrix for the influx of monocytes, fibroblasts, and keratinocytes into the wound area (Grinell, Billingham, & Burgess, 1981). In addition, fibrin is critical for cellular adhesion to the matrix as well as modulation of various cells within the wound.

Beyond hemostasis, a sanitary environment must be maintained at the site of injury. Numerous cells are involved in this function. Neutrophils are the first leukocytes to arrive at the wound site in significant numbers, due to their relatively high density in the bloodstream. In addition, numerous chemoattractants including fibrinopeptides, cleaved from fibrinogen and fibrin degradation products, are involved in the emigration of neutrophils to the injured tissue (T. J. Williams, 1988). Neutrophils destroy bacteria by both phagocytosis and enzymatic and oxygen radical mechanisms, causing additional tissue death and wound debridement (Clark, 1996; Elsbach & Weiss, 1992). If wound contamination has not occurred, neutrophil infiltration typically ceases within a few days. Neutrophils are then trapped within the wound clot and desiccated tissue, which is shed during later stages of wound healing. In addition, subsequent monocyte infiltration to the wound site further inhibits neutrophil emigration. (Clark, 1998). The remaining neutrophils are phagocytosed by macrophages within a few days (Newman, Henson, & Henson, 1982). However, if the wound site is contaminated, neutrophils will remain in the wound bed, potentially adversely affecting healing (Clark, 1998).

Monocytes, inflammatory cells critical for wound sterility, undergo phenotypic alterations to become macrophages in the presence of an inflammatory environment (Cohen, Diegelmann, & Lindblad, 1992). Macrophages continue to clean and debride the wound. Macrophages also secrete numerous cytokines including platelet derived growth factor (PDGF), fibroblast growth factor (FGF), and transforming growth factor-beta (TGF-β), all of which induce fibroblast migration and proliferation. In addition, macrophages release cytokines responsible for tissue formation (i.e., collagen production by fibroblasts) such as interleukin-4 (IL-4) (Clark, 1996). Therefore, monocyte-derived macrophages play a key role in the transition from inflammatory to reparative processes of wound healing. Macrophage-depleted

animals have defective wound repair characterized by incomplete wound closure (Leibovich & Ross, 1975).

As previously discussed, many cells participate in the inflammatory process via the synthesis of inflammatory mediators. These mediators (cytokines, chemokines, lipid mediators, etc.) are small molecules ranging form 8,000 to 40,000 daltons (Dinarello, 2000), which can be described as either proinflammatory or anti-inflammatory. The balance between the pro- and anti-inflammatory mediators (primarily cytokines) is thought to determine the outcome of healing. Furthermore, susceptibility to disease may be genetically determined by the balance between pro- and anti-inflammatory cytokine expression (Dinarello, 2000). The most widely-investigated pro-inflammatory mediators are interleukin-1 (IL-1) and tumor necrosis factor-alpha (TNF-α). IL-1 and TNF-α act synergistically to stimulate the expression of pro-inflammatory genes including type II phospholipase A_2, cyclooxygenase -2 (COX-2), and inducible nitric oxide synthase (iNOS) (Dinarello, 1997).

In contrast, anti-inflammatory mediators suppress pro-inflammatory gene expression, including expression of IL-1 and TNF-α. The inflammatory cascade is thus suppressed. The impact of anti-inflammatory cytokines can be most appreciated in the animal knockout model. For example, deletion of the IL-10 gene in mice results in fatal inflammatory bowel disease (Scheinin, Butler, Salway, Scallon, & Feldman, 2003). Also, inflammatory conditions such as rheumatoid arthritis are associated with deficiencies in IL-1 receptor antagonists (Isoda et al., 2003).

1.1.2. Tissue Formation

The process of tissue reformation at the site of injury can be further broken down into the reformation of the epithelial boundary and the formation of the sub-epithelial architecture.

Although these processes are interdependent, they are described separately for the sake of simplicity.

1.1.2.1. Reformation of the sub-epithelial architecture.

The main cell responsible for the reformation of the sub-epithelium is the fibroblast. In skin, fibroblasts migrate into the wound area from the surrounding tissue between 48 and 72 hours following injury (VanLis & Kalssbeek, 1973). The relative timing appears similar in the airway as well (Goldstein, Hebda, Klein, & Dohar, 1998). Although the specific chemotactic signals responsible for fibroblast migration remain relatively unknown, signals derived from macrophages in the wound bed are essential to facilitate fibroblast migration (Madtes et al., 1988). Furthermore, both platelet-derived growth factor (PDGF) and transforming growth factor-β have been shown to stimulate fibroblast migration *in vitro* (Postlethwaite, Keski-Oja, Moses, & Kang, 1987; Seppa, Grotendorst, Seppa, Schiffmann, & Martin, 1982). Fibroblast migration along the fibronectin matrix is achieved by reorganization and contraction of intracellular microfilaments. The direction of movement is determined by the alignment of the fibers in the provisional matrix. Furthermore, the presence of hyaluronic acid surrounding the matrix may facilitate fibroblast movement (Jacobson, O'Dell, Holifield, Murphy, & August, 1984).

Once at the wound site, fibroblasts have many roles in the wound healing process. Fibroblasts undergo several phenotypic changes in order to complete these functions, including migration (Clark, 1988). Fibroblasts produce large amounts of collagen and proteoglycans (Woodley, O'Keefe, & Prunieras, 1985). Type I collagen (the major matrix component in both the skin and the vocal folds) synthesis is stimulated by transforming growth factor-beta (TGF-β) secreted into the wound area (Varga & Jimenez, 1986). Furthermore, excessive TGF-β

production has been implicated in various fibrotic processes (Ma et al., 2003; Ozbilgin & Inan, 2003). Both glycosaminoglycans and proteoglycans are produced concomitantly with the production of collagen (Kirsner & Eaglstein, 1993). The signals responsible for glycosaminoglycan and proteoglycan production are relatively unknown. However, insight into the biochemical stimuli responsible for proteoglycan production, in particular, may be useful in anti-fibrotic therapy.

Fibroblasts also undergo a phenotypic alteration to become myofibroblasts, having characteristics of both smooth muscle cells as well as fibroblasts. Myofibroblasts are characterized by prominent rough endoplasmic reticulum required for increased production of matrix proteins associated with granulation tissue. Furthermore, myofibroblasts are characterized by cytoplasmic bundles of microfilaments, or stress fibers, which generate force homologous to smooth muscle cells. This process of phenotypic change to myofibroblasts is complex and poorly understood. However, environmental cues appear to stimulate fibroblast differentiation into proto-myofibroblasts, an intermediary cell type between fibroblasts and true myofibroblasts expressing α-smooth muscle actin (Tomasek, Gabbiani, Hinz, Chaponnier, & Brown, 2002). Growth factors and newly synthesized matrix materials are thought to then stimulate continued evolution of proto-myofibroblasts into differentiated myofibroblasts (Tomasek et al., 2002). Specifically, TGF-β appears primarily responsible for complete fibroblast differentiation into myofibroblasts (Desmouliere, Geinoz, Gabbiani, & Gabbiani, 1993).

Granulation tissue begins to form approximately four days after injury. This newly-synthesized tissue contains new vasculature, macrophages, myofibroblasts, fibroblasts, and a loose network of connective tissue (Clark, 1998). Contraction of the wound via myofibroblasts

is required to 1) maintain tissue continuity, 2) reduce the size of the wound, and 3) facilitate scar production (Gabbiani, 2003). Wounds contract by approximately 0.6 to 0.75 mm per day in a pig skin model (Larrabee, Bolen, & Sutton, 1988). Granulation tissue resolves with massive apoptosis of myofibroblasts when epithelialization is complete (Desmouliere, Redard, Darby, & Gabbiani, 1995). In addition, it has been hypothesized that myofibroblasts revert back to a quiescent form upon wound closure (Desmouliere, 1995). However, failure to down-regulate myofibroblast activity has been implicated in fibrotic processes.

1.1.2.2. Re-epithelialization.

At the most basic level, the major goal of healing is the reconstitution of the epithelium as a functional barrier. Clinically, a wound is re-epithelialized when a water-impermeable seal is created (Hackam & Ford, 2002). In skin, epidermal growth factor and fibroblast growth factor stimulate keratinocyte migration to the site of injury (Martin, 1997; Werner, 1998). This process typically begins 24 to 48 hours after injury (O'Toole, 2001).

In order for epithelial cells to migrate, they must detach from surrounding cells and the underlying basement membrane zone. Migration is characterized by the extension of a "foot" or lamellipod, followed by advancement of the epithelial cell body. Following lamellipod extension, retraction of the rear of the cell propels cell movement (Rilla et al., 2002). The fibronectin-rich provisional matrix appears to be essential for epidermal cell migration. The provisional matrix enables keratinocytes to proceed under the eschar and debris covering the wound (Grove, 1982). Separation of the fibrinous crust from the underlying tissue is enhanced by epidermal cell release of proteases, including matrix maetalloproteinase-1 and plasmin. Plasmin then promotes the resolution of the eschar along the path of the migrating epithelial cells

to replace the clot with reconstituted epithelium (Arumugam, Jang, Chen-Jensen, Gibran, & Isik, 1999).

1.1.3. Tissue Remodeling

Wound healing and tissue repair continue long after a functional epidermal barrier has been restored. These processes are collectively referred to as the remodeling phase of wound healing and involve the deposition and re-organization of matrix materials over time. Scar remodeling is typically thought to be the final stage of the wound healing cascade. Early scar, between one to three months following injury, is thick and stiff. In contrast, more mature scar is typically more thin and pliable. Scar pliability has significant implications for tissue mobility. The differences between immature and mature scar are thought to be due, at least in part, to the reorganization of collagen fibers along lines of stress as well as changes in proteoglycan content (Ehrlich, 2000). Collagen content has been found to become relatively stable approximately 21 days following injury. However, both collagen deposition and remodeling are dynamic processes (Ehrlich, 2000; Martins-Green, 1997) and are thought to continue up to 12 months following injury.

In the dermatology literature, two types of excessive scarring have been described: hypertrophic scars and keloids. Hypertrophic scars are raised lesions that do not go beyond the original wound edges. In contrast, keloids extend beyond the wound edges (Tredget, Nedelec, Scott, & Ghahary, 1997). Both types of scars are characterized by an increased inflammatory response and ECM production (Bettinger, Yager, Diegelmann, & Cohen, 1996). Keloids are characterized by large, thick collagen fibers composed of numerous fibrils packed closely together. In contrast, hypertrophic scars exhibit nodular structures containing cells, vessels, and fine, randomly organized collagen fibers (Ehrlich et al., 1994). In addition, all scars are

characterized by increased collagen type I and decreased collagen type III, glycosaminoglycans, and water (Ehrlich, 2000).

In vitro, keloid fibroblasts produce markedly increased levels of collagen, fibronectin, elastin, and proteoglycans when compared to normal dermal fibroblasts (Babu, Diegelmann, & Oliver, 1992). Fibroblasts from hypertrophic scars also produce elevated levels of collagen. However, metabolic modulators (glucocorticoids, hydrocortisone, and growth factors) affect hypertrophic fibroblasts and normal fibroblasts similarly; in contrast, keloid fibroblasts display aberrant responses to such modulators. Taken together, experimental evidence suggests that keloid and hypertrophic scar fibroblasts are phenotypically different from normal fibroblasts, and as interestingly, different from each other (Bettinger et al., 1996; Gadson, Russell, & Russell, 1984).

In vivo, tension on the wound edge has been implicated in both keloid and hypertrophic scar formation. Increased tension within the wound is thought to alter fibroblast phenotype expression (Younai et al., 1996). Therefore, both keloids and hypertrophic scars are more common in wounds closed under tension. For example, sites under high skin tension such as the shoulder and presternal area develop keloids and hypertrophic scars at increased frequency compared to other sites.

1.2. Wound Healing in the Upper Airway

Although not as extensively studied as the skin, the wound healing process in the subglottis has recently been characterized. Given the relative proximity of the subglottis to the vocal folds, a review of the upper airway healing literature is relevant. In general, it appears that the wound healing process in the airway occurs in similarly delineated phases as for the skin. However, epithelialization appears to be prolonged in the subglottis due to the presence of

epithelial reservoirs, which may alter the process (Goldstein et al., 1998). In addition, instead of eschar described in the skin, a fibrinous clot is formed in the airway (Goldstein et al., 1998).

Injury to the upper airway may yield less than desirable outcomes in the long-term. Squamous metaplasia of the airway epithelium decreases mucociliary function and places the tissue at increased risk of future injury (Duynstee, de Krijger, Monnier, Verwoerd, & Verwoerd-Verhoef, 2002). Subglottic stenosis, a relatively common narrowing of the subglottic lumen associated with prolonged endotracheal intubation, is characterized by fibrous scar of the lamina propria with projections into the lumen due to irregular granulation formations. In addition, irregularities of the tracheal rings with cellular advancement have been reported. Injury to the perichondrium may increase the likelihood of stenosis (Duynstee et al., 2002). Dohar, Klein, Betsch, and Hebda (1998) confirmed that the depth of injury, not the lateral extent, is the key predictive factor in acquired subglottic stenosis.

Regardless of the relative proximity of the subglottis to the vocal folds, marked anatomic and physiologic differences limit the generalizablity of the characteristics of wound healing from the subglottis to the vocal folds. Therefore, a thorough review of the anatomic constituents and wound healing process in the vocal folds is warranted.

2. Vocal Fold Architecture

The vocal folds are unique in both structure and function. A review of the microarchitecture of the vocal folds is necessary to think clearly about the questions posed in the present investigation. To that end, the following text reviews many of the historic studies in addition to current work regarding vocal fold structure. For simplicity's sake, the text is organized according to anatomical structure. Specifically, separate sections are dedicated to the epidermis and basement membrane, lamina propria (both cellular and extracellular components), and macula flava.

2.1. Epithelium and Basement Membrane

Under electron microscopy, the surface of the vocal folds appears corrugated due to the presence of microvilli (S. D. Gray, Titze, & Lusk, 1987). Microvilli have been described in relatively few regions of the body. Specifically, microvilli have been isolated to regions that require significant lubrication such as the cornea (Castrow & Krul, 1983). Gray and colleagues (1987) suggested that microvilli of the vocal folds function as "treads of a tire," trapping and spreading mucus evenly across the surface of the vocal folds. In addition, microvilli may serve to increase the surface area of the vocal folds, aid in hydration, and provide "traction" during vocal fold vibration (S. D. Gray, 2000; S. D. Gray et al., 1987). Destruction of microvilli has been associated with hyperphonation in a canine model due to superficial injury during phonation (S. Gray & Titze, 1988).

Within the vocal fold epithelium, two distinct cell populations have been identified. The deepest layer is composed of basal cells (S. D. Gray, Pignatari, & Harding, 1994). Basal cells are characterized by their position within the epithelium as well as by the presence of hemidesmosomes (Evans, Van Winkle, Fanucchi, & Plopper, 2001). Basal cells of the vocal fold have been largely overlooked and are poorly described in the literature. However, the relative position of basal cells, which are sandwiched between the overlying epithelial cells, basement membrane, and underlying mesenchymal cells suggests an important role in epithelial - mesenchymal communication (Evans & Moller, 1991). Tracheal basal cells have been implicated as a major component of the epithelial-mesenchymal unit facilitating intercellular communication (Evans, Van Winkle, Fanucchi, & Plopper, 1999). This function of basal cells has not been confirmed in the vocal folds.

The superficial epithelium is composed of stratified squamous epithelium cells. The epithelium of the vocal folds is contiguous with the ciliated epithelium of the ventricular folds

and trachea as well as the stratified columnar epithelium of the epiglottis. The epithelium serves to protect and contain the underlying lamina propria. Desmosomes are responsible for intercellular adhesion allowing the epithelium to remain intact despite the mechanical trauma associated with phonation (S. D. Gray et al., 1987). Desmosomal concentration is decreased in the most superficial layers of vocal fold epithelial cells, allowing for normal exfoliation. In contrast, desmosomes are more developed in somewhat deeper layers of the vocal fold epithelium (S. D. Gray et al., 1987). Desmosome junctional complexes have been shown to be critical in intercellular signal transduction in other tissue (Green & Gaudry, 2000). This concept has not been investigated in the vocal folds.

A layer of mucus, the mucociliary blanket, covers the epithelium providing hydration to the underlying epithelial cells. The mucociliary blanket is composed of a mucinous layer and a serous layer. The mucinous layer is more superficial and serves to protect the serous layer as well as the underlying cells, and is composed of various types of mucin molecules. The serous layer is in contact with epithelial cells and primarily contains water. Other constituents of the serous layer have not been identified (S. D. Gray et al., 1987; Kobayashi & Wanner, 1993). Airway cilia beat the mucociliary blanket up from the trachea at a rate of 4 to 21 mm/min in healthy adults, keeping the vocal folds hydrated under normal conditions (S. D. Gray et al., 1987; Sacker, Landa, Hirsch, & al, 1975; Wanner, 1977)

The vocal fold epithelium is connected to the lamina propria via anchoring fibrils of the basement membrane zone (BMZ). The basement membrane zone is a collection of proteins as well as non-protein structures that aid in securing the basal cells to the extracellular matrix of the lamina propria and appear to have ultrastructural patterns similar to the skin (S. D. Gray et al., 1994). In addition to governing epithelial-lamina propria adherence, the BMZ is also responsible

for giving shape to the underlying lamina propria (S. D. Gray, 2000). Anchoring fibrils composed of collagen type VII and fibronectin are the main structural components of the BMZ (Courey, Shohet, Scott, & Ossoff, 1996; Sakai, Keene, Morris, & Burgeson, 1986). Anchoring fibrils loop from the lamina densa of the BMZ into the lamina propria, looping around collagen type III fibers, and then back into the lamina densa (S. D. Gray et al., 1994). Anchoring fibril deficiencies have been implicated in the failed epidermal-dermal adherence in diseases such as epidermolysis bullosa dystrophica (Briggaman & Wheeler, 1975). The density of anchoring fibrils in the vocal folds is unknown. In the skin, significant individual variation has been reported. Tidman and Eady (1984) found anchoring fibril density to be greatest in regions of the body subjected to large shearing and mechanical stress.

The role of the epithelial-BMZ complex in phonation appears substantial. Disruption of anchoring fibrils has been implicated in vocal fold nodules and other benign pathological lesions of the vocal folds (Courey et al., 1996; S. Gray, 1989). In vocal nodules, anchoring fibrils are partially formed, poorly organized, and do not always link the lamina densa to the lamina propria (S. Gray, 1989). Improved characterization of these structures could lead to improved management strategies for dysphonia. Interestingly, Gray and colleagues suggested that anchoring fibril density in the vocal folds may be genetically determined (S. Gray, 1989). Thus, some humans may have a predisposition for certain forms of vocal fold pathology.

2.1.1. Lamina Propria

The lamina propria (LP) of the vocal fold is an area of connective tissue situated between the epithelium and thyroarytenoid muscle. The LP is a relatively small area with high concentrations of cell-secreted matrix substances such as glycosaminoglycans (GAGs), proteoglycans, and fibrous proteins (collagen and elastin). Typically, the LP is described by the

relative depth of the tissue: superficial, intermediate, and deep. The present review further bisects the LP into cellular and extracellular components. The layered structure will be discussed throughout. The macula flava will be discussed separately as it has distinct cellular and connective tissue characteristics.

Hirano delineated the lamina propria of the vocal folds as having three layers based on connective tissue density (M. Hirano, 1975, 1977). The superficial layer of the lamina propria (SLP), or Reinke's space, contains loose tissue with few collagen or elastin fibers. Fibrous protein concentration increases with increasing depth of the lamina propria. The cover/body theory of vocal fold vibration is based on the varying densities of fibrous proteins associated with the layered structure of the lamina propria. The epithelium and SLP compose the "cover", whereas the deep layer of the lamina propria and vocalis muscle make up the body. The intermediate layer of the lamina propria is considered transitional (M. Hirano, 1975, 1981). Furthermore, the intermediate and deep layers of the lamina propria together constitute the vocal ligament (an anatomical misnomer), which is thought to bear most of the mechanical stress associated with phonation (M. Hirano & Kakita, 1985).

The relative depth of the SLP varies along the length of the vocal fold. This layer is the thickest at the midpoint of the musculomembranous vocal folds and becomes thinner towards the anterior and posterior regions. Conversely, the intermediate layer is thinnest at the mid-fold region and becomes thicker near the ends. The thick regions of the intermediate layer of the lamina propria proximal to the articulation with the vocal process and thyroid cartilage are referred to as the anterior and posterior macula flava, discussed shortly. The deep layer is thickest in the posterior region of the vocal folds. These length-wise structural variations are

thought to provide "cushion" to protect the anterior and posterior margins of the vocal folds from mechanical damage associated with phonation (M. Hirano, Kurita, & Nakashima, 1981).

2.1.1.1. Cellular components of the lamina propria.

Most of the literature on the cellular content of the vocal folds has focused on fibroblasts. However, the lamina propria may be maintained by several different cells, not just fibroblasts (Catten, Gray, Hammond, Zhou, & Hammond, 1998; Pawlak et al., 1996). Macrophages, myofibroblasts, and fibroblasts have all been localized within the lamina propria of human vocal folds. However, the distribution of the three cell types appears irregular. Fibroblasts, the most abundant cell type in the lamina propria, have been found throughout the entire depth of the LP. However, increased fibroblast density was noted in the deepest 20% of the lamina propria. Increased fibroblast concentration corresponds to increased collagen density in the deep layer of the LP.

Macrophages, which are inflammatory cells, were isolated in approximately one third of LPs in a study by Catten and colleagues (1998). As a component of the inflammatory process, macrophages are responsible for maintaining wound sterility and the secretion of inflammatory mediators. Interestingly, macrophages were localized to the most superficial 20% of the lamina propria. The authors suggested that macrophages were a part of the mucosal response to environmental irritants (smoking, pollution, workplace contaminants). Therefore, macrophage presence may indicate pre-mortem exposure to inflammatory stimuli (Catten et al., 1998).

In that study, unlike macrophages, all the samples examined contained myofibroblasts. Also, these cells were most abundant in the most superficial 20% of the lamina propria The authors suggested that myofibroblasts may function to repair constant or ongoing injury incurred through normal use of the vocal mechanism (Catten et al., 1998). This hypothesis correlates well

with the general thought that myofibroblasts function in both periodic and continual reorganization of tissue (Sappino, Schurch, & Gabbiani, 1990).

Cellular density, and more specifically, fibroblast density within the lamina propria appears to be age-specific. Fibroblast density in both the LP and the macula flava is significantly higher in newborns and children than in adults (M. Hirano & Kakita, 1985; Sato & Hirano, 1995a). Fibroblast morphology and protein production also varies significantly across the life-span. In the macula flava, more active fibroblasts with developed rough endoplasmic reticulum and Golgi apparatus have been found in adults compared to newborns. Within the lamina propria, inactive fibroblast density appears increased in newborns (M. Hirano, Sato, & Nakashima, 1999). Increased density of inactive fibroblasts in the newborn lamina propria is suggestive of an active proliferative phenotype with minimal ECM synthesis (M. Hirano et al., 1999). In contrast, decreased fibroblast density has been reported in the geriatric (74-83 years old) macula flava when compared to the macula flava of younger adults (34-51 years old). Interestingly, in spite of differences in the MF, the density of fibroblasts in the lamina propria was not different between the two age groups. These findings suggest a substantive role for the macula flava not only in early development, but also in vocal fold aging (M. Hirano, Sato, & Nakashima, 2000).

2.1.1.2. Extracellular matrix.

Gray, Titze, and Lusk (1988) suggested that the relative sparseness of cells within the lamina propria indicates that the extracellular matrix (ECM) has the prevailing role in vocal fold vibration. In general, the ECM is composed of an organized network of macromolecules that determine the physical properties of the tissue (Alberts, 1999). Historically, the ECM was thought only to support the overlying epithelium. However, more recent thinking suggests that

the ECM is critical for cell adhesion, migration, growth, and differentiation, as well as for the modulation of cytokines and growth factors (Parsons-Wingerter & Sage, 1997). Although most of the literature has focused on the structural role of the vocal fold ECM, cell-matrix signaling warrants investigation and may yield insight into the dynamic nature of matrix formation and response to injury.

The extracellular matrix (ECM) is composed of fibrous proteins (collagen and elastin), structural glycoproteins (fibronectin and laminin), glycoaminoglycans (hyaluronic acid), proteoglycans, and other interstitial molecules such as carbohydrates and lipids (S. D. Gray, Titze, Chan, & al, 1999; Labat-Robert, Bihari-Varga, & Robert, 1990). Due to methodological challenges, little is known about the presence or role of carbohydrates and lipids within the ECM of the vocal folds. Therefore, these components will not be further discussed due to the current paucity of literature.

Both collagen and elastin provide structure and define the major biomechanical attributes of vocal fold tissue. Collagen is critical in bearing stress and resisting deformation of the tissue during phonation (S. D. Gray, 2000). In contrast, elastin allows tissue to return to its original shape following deformation (S. D. Gray, 2000).

Collagen types I, II, and III have been identified within the lamina propria of the vocal folds (S. D. Gray, Hirano, & Sato, 1993). Collagen fibers within the LP are oriented longitudinally from the anterior to the posterior LP. This orientation is thought to allow the tissue to withstand longitudinal stress associated with vocal fold oscillation (S. D. Gray, Titze, Alipour, & Hammond, 2000). Scanning electron microscopy of the vocal fold lamina propria following formic acid treatment revealed clusters of collagen fibers in the superficial layer, thick

bundles of collagen fibers in the intermediate layers, and dense collagen bundles in the deep layer (Ishii, Zhai, Akita, & Hirose, 1996).

Elastin is present in three structural entities in the lamina propria of the vocal fold: elaunin, oxytalan, and mature elastin (Hammond, Gray, Butler, Zhou, & Hammond, 1998). Oxytalan is composed of microfibrils 10 to 12 nm in diameter. Elaunin is composed of both microfibrils and a small amorphous component. Elastin fibers, sometimes referred to as mature elastin, have larger amorphous components as a core and microfibrils surrounding the core. Elastin fibers have the most elastic characteristics, and can be stretched to approximately two times their original length and then return to their pre-stretch dimension (Porto, Chevalier, Peyrol, Guerret, & Grimaud, 1990). Elaunin and oxytalan are less pliable and are found in tissues such as tendons and cartilage that withstand high stress (Ferreira, Caldini, & Montes, 1987).

Elastin concentration dramatically increases approximately 25% into the lamina propria, and begins to level off at approximately 45%. Elastin density remains high until the last 15% to 20% of the lamina propria (S. D. Gray et al., 2000). Although the layered structure of the LP is typically described in terms of collagen content, elastin concentration may also provide some insight into the biomechanical properties of the vocal folds. The superficial layer of the lamina propria consists of approximately 25% to 35% of the vocal fold based solely on elastin distribution (S. D. Gray et al., 2000). The intermediate layer of the lamina propria makes up the next 45% to 55% of the entire depth of the lamina propria, and is characterized by extensive, mature elastin fibers. The deep layer of the lamina propria makes up the deepest 20% of the lamina propria with a significant decrease in elastin concentration in the deepest stratum.

Although there may be some question regarding the distribution of collagen within the LP, elastin content within the LP determines the classic tri-layered vocal fold structure.

Structural glycoproteins, proteoglycans, and glycoaminoglycans are located in the spaces between fibrous proteins and may bind fibrous proteins influencing fiber mechanics (Pawlak et al., 1996). Interestingly, non-fibrous proteins have a layered orientation similar to fibrous proteins in the vocal fold lamina propria, and as such may influence the mechanical properties of the vocal folds (S. D. Gray, Titze, Chan, & Hammond, 1999). Non-fibrous or interstitial proteins control tissue viscosity, water content, tissue size and thickness, and the size and density of collagen fibers (S. D. Gray et al., 2000; S. D. Gray, Titze, Chan, & Hammond, 1999).

Hirschi, Gray, and Thibeault (2002) found fibronectin to be ubiquitous throughout the vocal fold lamina propria. The function of fibronectin remains largely unknown but it is thought to bind proteins and provide strength and adhesion between molecules (Labat-Robert et al., 1990). Increased fibronectin concentration has been found in both nodules and vocal fold scar, and could be a target of anti-fibrotic therapy (Courey et al., 1996; S. D. Gray, 1997).

Three subgroups of proteoglycans have been identified within the ECM of the vocal folds: 1) small, interstitial matrix proteoglycans such as decorin, biglycan, and fibromodulin, 2) large, aggregating chondroitin sulfate proteoglycans including aggrecan and versican, and 3) heparin sulfate proteoglycans (Pawlak et al., 1996). Proteoglycans consist of a protein core with a linkage protein to attach to the cores of other proteins, carbohydrates, and lipids (Ruoslahti, 1988). Proteoglycans can, therefore, transport molecules with varying biological characteristics and affinities, including water (S. D. Gray, Titze, Chan, & al, 1999). In addition, proteoglycans seem to aid in the regulation of the biological performance of other matrix proteins (S. D. Gray, Titze, Chan, & Hammond, 1999). Proteoglycans also bind growth factors such as fibroblast

growth factor (FGF) to facilitate receptor activation (Turnbull, Fernig, Ke, Wilkinson, & Gallagher, 1992). In contrast, decorin sequesters TGF-β, a fact that may have significant biomechanical implications (Gumienny & Padgett, 2002).

All three types of proteoglycans have been identified within the LP of the vocal folds. Versican has been localized to macrophages and fibroblasts (Pawlak et al., 1996). Versican binds hyaluronic acid and water to fill space within the lamina propria (Lander, 1993; Zimmerman, 1993). Versican is vital to maintain adequate hydration in other tissues, such as cartilage (Lander, 1993). Aggrecan was not found in the lamina propria of the vocal fold (Pawlak et al., 1996). The small interstitial proteoglycans, decorin and fibromodulin have been found in the superficial and intermediate layers of the vocal fold lamina propria, respectively. Both of these molecules are thought to bind collagen and delay fibril formation and also induce the formation of thinner fibrils yielding optimal biomechanical tissue (Hardingham & Fosang, 1992). Heparan sulfate has been found in both macrophages and fibroblasts in the vocal fold lamina propria and also in the basement membrane zone. Heparan sulfate is thought to bind to fibronectin, collagen, and laminin and is a critical component of the basement membrane (Heremans, DeCock, Cassiman, Van den Berghe, & David, 1990).

Of particular relevance to the current study, Gray, Titze, Chan, and Hammond (1999) hypothesized that decorin in the superficial lamina propria explains the minimal scarring associated with phonomicrosurgery involving only the SLP. In fact, decreased decorin content has been implicated in vocal fold scar (Thibeault, Gray, Bless, Chan, & Ford, 2002). Therapy targeting proteoglycan synthesis and specifically increased decorin synthesis will likely be an anti-fibrotic treatment of the near future.

Only one glycosaminoglycan (GAG), hyaluronic acid (HA), has been identified in the vocal fold lamina propria. Gray and colleagues (1999) suggested that HA is a biomechanically ideal architectural component of the vocal fold, allowing the tissue to withstand vibratory collisions. HA is also critical to maintain optimal tissue viscosity in the vocal folds (Chan, Gray, & Titze, 2001). Hyaluronic acid differs from proteoglycans in that it is not covalently attached to a protein core, and is considered a separate, unique entity (Lander, 1993). HA is porous, loosely folded, and globular (Laurent, Laurent, & Fraser, 1995). HA is also a relatively inflexible, hydrophilic molecule which is thought to provide the "space-filling" characteristic required to resist compressive forces in the vocal folds (S. D. Gray, 1997, 2000). A single HA molecule takes up 1000 times more space than expected based on its molecular weight and composition (Laurent et al., 1995). Interestingly, men have a threefold higher HA content within the LP, as compared to women, which may be the reason that many more women present with vocal pathology associated with phonotrauma (Hammond, Zhou, Hammond, Pawlak, & Gray, 1997).

2.2. Macula Flava

The vibratory portions of the vocal folds are connected to the anterior commisure of the vocal fold with the thyroid cartilage via the anterior macula flava (MF). Posteriorly, the vocal folds articulate with the vocal process of the arytenoid cartilage bilaterally through the intervening posterior macula flava (M. Hirano & Sato, 1993; Sato & Hirano, 1995b). The MF are yellowish regions of dense fibrous tissue composed of elastic fibers, collagenous fibers, and fibroblasts (Sato & Hirano, 1995b). The MF are elliptical and approximately 1.5 x 1.5 x 1.0mm in diameter (Sato & Hirano, 1995b). Three-dimensional imaging reveals collagenous fibers, reticular fibers, and elastic fibers running in various directions within the macula flava, and

projecting into the lamina propria of the vocal fold. HA has been localized to the space among the fibers of the MF possibly implicating enhanced shock absorption (K. Sato, M. Hirano, & T Nakashima, 2003a). The macula flava appear to play key roles not only in phonation, but also in development, growth, and aging of the vocal folds (Sato & Hirano, 1995b).

Historically, the MF was thought useful to avoid damage to the ends of the vocal fold associated with phonation (M. Hirano, 1975). Beyond shock absorption, the MF are currently thought to be involved in vocal ligament synthesis (Sato & Hirano, 1995b). Sato and Hirano (1995b) reported alignment of fibroblasts in the direction of the vocal ligament at the transition between the MF and the vocal fold. Mechanical stress associated with phonation causes fibroblasts within the MF to become active, and synthesize fibrous proteins (M. Hirano et al., 1999; Sato & Hirano, 1995b).

Interestingly, newborns lack a vocal ligament. However, newborns have immature MFs in the same position as adult MFs (M. Hirano, Kurita, & Nakashima, 1983; M. Hirano & Sato, 1993). Phonation after birth is thought to stimulate cells in the MF to produce the fibrous components of the vocal ligament (Sato & Hirano, 1995a, 1995b). Sato, Hirano, and Nakashima (2003a) suggested that the MF are critical to ECM metabolism, determining the viscoelastic properties of the human vocal fold throughout the lifespan (Sato et al., 2003a).

In addition, a unique cell type has been identified in the MF, vocal fold stellate cells (VFSC). These cells are not found in the LP and differ from fibroblasts in both shape and function. Fibroblasts in the LP have large a nucleus-to-cytoplasm ratio and less developed rough endoplasmic reticula, suggesting inactivity under normal conditions. In addition, the ECM surrounding conventional fibroblasts is sparse. Fibroblasts may be activated in a pathological state such as injury, but remain relatively inactive in the normal state (K. Sato, M. Hirano, & T.

Nakashima, 2003b). In contrast, VFSCs are smaller and have slender cytoplasmic processes (M. Hirano et al., 1999; Sato, Hirano, & Nakashima, 2001). VFSCs also contain well-developed rough endoplasmic reticula and Golgi apparatuses suggesting continual, active protein synthesis. VFSCs are surrounded by dense extracellular materials within the MF, suggesting that VFSCs constantly synthesize ECM materials under normal conditions (Sato et al., 2001).

VFSCs also contain cytoplasmic lipid droplets that store vitamin-A. Vitamin-A induces transforming growth factor-β (TGF-β) thereby stimulating ECM synthesis (Glick, Flanders, Danielpour, Yuspa, & Sporn, 1989; Sato et al., 2003b). Observations regarding the presence of vitamin-A storing droplets may contribute to suggestions that VFSCs are responsible for continuous matrix formation. VFSCs appear homologous, in both appearance and function, to hepatic stellate cells (HSC) (Sato et al., 2003b). HSC activation is the central event associated with hepatic fibrosis (Safadi & Friedman, 2002). Further characterization of VFSCs may yield insight into fibrotic processes in the vocal folds.

3. Wound Healing in the Vocal Folds

The vocal folds, unlike most other tissues in the body, are subjected to continuous mechanical stresses due to phonation. Given this mechanically stressful state, it is surprising that vocal folds are not structurally and physiologically compromised more often than they are. At least two possible explanations are possible for this resistance to mechanical stress. The first is that the vocal folds have an enhanced reparative capacity, by which microstructural damage to the lamina propria and overlying epithelium can be repaired quickly and without a full-scale wound healing response. The second explanation is that the microstructure of the vocal fold lamina propria is organized to accommodate more mechanical stress than tissue such as skin. It is likely that both hypotheses are correct. In addition, if vocal folds can accommodate some level of continuous mechanical stress, it stands to reason that a threshold would exist, past which

a full-scale wound healing response is required for tissue repair. The following review categorizes vocal fold injury into four types based on the source and chronicity of the injury: acute phonotrauma, chronic phonotrauma, non-phonatory mechanical injury, and chemical injury.

3.1. Acute Phonotrauma

Acute phonotraumatic injury involves vasodilation and subsequent leakage of plasma into the extravascular compartment (Courey et al., 1996). Beyond disruption of the vascular network, data suggest that the basement membrane zone and extracellular matrix may also be affected. Clinically, acute phonotrauma manifests as edema of the vocal folds, or laryngitis. In addition, many patients may present with focal regions of acute inflammation at the midpoint of the musculomembranous vocal folds. This region is the site of greatest impact stress during phonation (Jiang & Titze, 1994). Mass lesions may develop with continued phonotrauma to the region. Frequently, edema associated with phonotrauma resolves over time and voice quality is improved as a result.

A description of the magnitude and temporal pattern of vocal fold inflammation may help elucidate the subsequent pathological events resulting in long-term vocal fold damage. Recent studies have described the acute inflammatory response in the vocal folds by measuring levels of inflammatory mediators in vocal fold surface secretions. Marked shifts in interleukin-1β (IL-1β), tumor necrosis factor-α, and matrix metalloproteinase-8 levels have been reported following an episode of acute phonotrauma (Verdolini, Rosen, Branski, & Hebda, 2003a). These data may prove useful for future investigations into the acute wound healing response in the vocal folds.

3.2. Chronic Phonotrauma

Multiple episodes of acute phonotrauma can result in long-standing tissue damage. Although they are often termed chronic phonotrauma, these episodes quite likely encompass many of the events present in a single acute episode of phonotrauma. The result is a relatively permanent state of tissue repair or scarring, which may manifest as a benign vocal fold lesion or vocal fold scar. It is also likely that there is a continuum of responses associated with chronic phonotrauma whereby focal edema gives way to mass lesions and finally, vocal fold scar based on the chronicity of injury. Vocal fold scar should not be thought of in terms of more commonly encountered dermal or mucosal scars. Whereas the dermis and airway mucosa are relatively static structures, the vocal folds are subjected to mechanical stress. As a result, the process of chronic vocal fold scarring is likely to be quite different from processes encountered in other tissues. The literature described three general types of long-standing, structural abnormalities of the vocal folds: nodules, polyps and cysts. Studies regarding vocal fold lesions are limited due to a lack of standard nomenclature (Rosen & Murry, 2000). In addition, the extent to which each of these pathologies is truly "scar" is yet unclear. Furthermore, a continuum of vocal fold scar may exist from scar proper to sulcus vocalis.

Vocal fold nodules are likely the most commonly diagnosed lesion of the vocal folds and are thought to be a consequence of repetitive vocal fold trauma. Nodules involve a disruption of the basement membrane zone with separation of the epithelium from the underlying extracellular matrix (S. D. Gray, Hammond, & Hanson, 1995). Kotby and colleagues (1988) described vocal fold nodules as having intercellular junction gaps, disruption and some duplication of the basement membrane zone, and focal collagen deposition within the SLP. Increased levels of fibronectin have also been identified in the SLP at sites of nodular lesions (Courey et al., 1996). It has been hypothesized that the disruption of the basement membrane zone associated with

vocal fold nodules may place the tissue at increased risk for repeated injury, possibly yielding vocal fold stiffness or scar (S. D. Gray et al., 1995).

Polyps are thought to be more of an acute vascular injury characterized by less fibronectin deposition and basement membrane zone disruption (Courey et al., 1996). Vocal fold polyps have also been described as hemorrhage, fibrin and iron deposition, and thrombosis in the SLP (Dikkers & Nikkels, 1995). Kotby and colleagues (1988) proposed that nodules and polyps represent a relative continuum of vocal fold injury with critical variables being the chronicity of the wound and the focal or diffuse nature of the injury. Similar to vocal fold edema, polyps are likely a relative arrest in the wound healing process. In contrast, mature nodules are likely a result of complete wound healing with deposition of fibronectin as a precursor to scar formation.

Vocal fold cysts are typically less common than other benign vocal fold lesions. In addition, cysts of the true vocal folds are less common than other laryngeal cysts (Shvero et al., 2000). It remains unclear if cysts are a result of a reparative process associated with phonation. Courey and colleagues (1996) described cysts as having a basement membrane zone thickness between that of polyps and nodules. In addition, there may be different types of cysts with varying cell type lining of the lesion. Cysts may be lined with either columnar or squamous epithelium (Shvero et al., 2000). The implications for these findings remain unclear. However, it is speculated that cysts located at the midpoint of the membranous vocal folds are likely due, at least in part, to injury associated with high phonatory vocal fold impact stress.

As for acute vocal fold injury, the assay of biochemical markers associated with chronic vocal fold lesions may provide some insight into the nature of these lesions. Patients with active epithelial disease such as recurrent respiratory papilloma and squamous cell carcinoma present with markedly increased levels of IL-1β, suggesting an active inflammatory response to invasive

disease processes (Branski, Verdolini, Rosen, & Hebda, 2004). In contrast, patients with chronic pathological conditions of the lamina propria (cysts and polyps) do not appear to have significant IL-1β upregulation (Branski et al., 2004). These data suggest that benign vocal fold lesions represent the end-product of the wound healing cascade, or at least an arrest of the process beyond the acute, active inflammatory state (Branski, Verdolini, Rosen, & Hebda, 2003). In addition, patients with established lamina propria lesions present with increased prostaglandin-E2 (PGE-2) levels (Branski et al., 2004). PGE-2 is an inflammatory mediator and relatively ubiquitous in wound healing. Increased PGE-2 expression appears to differentiate established lesions from acute vocal fold injury from and may prove to be useful as a means to describe vocal fold injury (Branski et al., 2004).

3.3. Non-Phonatory Mechanical Injury

The vocal folds withstand regular mechanical trauma during phonation. However, the folds are also subjected to a myriad of non-phonatory sources of mechanical injury. Examples are endotracheal intubation and phonomicrosurgery, which result in a wound healing response.

Phonomicrosurgery is common for the removal of vocal fold lesions and can result in disruption of the epithelium, basement membrane and underlying lamina propria depending upon the depth of the incision. In the most severe case, complete ablation of the mucosa, a fibrinous clot forms at the site of the wound within one day following injury, in a rabbit model. By three days following injury, sparse epithelial coverage is present. More important, massive cellular infiltration and neo-lamina propria deposition is also noted by three days post injury. Complete epithelial coverage is achieved by five days following injury. Neo-lamina propria deposition increases substantially seven days following injury. By 21 days following surgical injury, the tri-

layered structure of the lamina propria is typically not regained (Rosen, Branski, Verdolini, & Hebda, 2003). It is unclear whether the layered structure is ever regained.

The precise sequence of events that follow surgical injury of the vocal fold can only be elucidated using appropriate animal models. Decreased collagen density in the scarred vocal fold as compared to normal folds was reported 60 days following forcep biopsy taken from the midpoint of the membranous vocal folds in a rabbit model (Thibeault, Gray et al., 2002). Thibeault and colleagues (2002) suggested that scar in the vocal folds is associated with a loss of normal collagen architecture, not increased collagen density. However, that investigation may describe "immature" scar as new matrix materials may not be stable until beyond 60 days. In addition, increased procollagen I (a triple-helix precursor to collagen typically used to measure newly formed collagen in fibrotic tissue) was noted in the superficial lamina propria of the injured vocal folds.

The concentration of many ECM components following vocal fold surgical injury has been described. Decreased elastin content has been reported 60 days following injury. Elastin in scarred vocal folds is composed of short, compact fibers. Although hyaluronic acid has been found predominately in the deep lamina propria of uninjured vocal folds, in one surgical study, HA was distributed throughout the scarred lamina propria, likely as a wound healing response mechanism. In that study, no significant difference was found between HA density in scarred versus normal vocal folds (Thibeault, Gray et al., 2002).

Thibeault, Bless, and Gray (in press) found significantly decreased levels of decorin 60 days following surgical injury in a rabbit model. Decorin, a proteoglycan found primarily in the superficial layer of the lamina propria, binds collagen and alters the kinetics of fibril formation (Pawlak et al., 1996). Decreased levels of decorin have been reported in hypertrophic scar in the

skin (Sayani et al., 2000; P. G. Scott, Dodd, Tredget, Ghahary, & Rahemtulla, 1995). Decreased decorin levels may be responsible for the altered collagen ultrastructure and resultant dysphonia associated with vocal fold scar. In skin, decorin increases to subnormal levels over time following burn injuries (Sayani et al., 2000). It is unclear if this process takes place in the vocal folds. However, germane to the present investigation, it is likely that decorin levels increase with time as well as with *tissue motion*. In fact, decorin content in other tissues has been shown to increase with exercise. Decorin is an interesting vocal fold protein that warrants investigation as a potential anti-fibrotic agent.

In addition, decreased levels of fibromodulin have been found in vocal fold scar 60 days following injury (Thibeault et al., in press). Fibromodulin has been shown to inhibit transforming growth factor-beta (TGF-β)-induced collagen synthesis. Decreased levels of fibromodulin have been implicated in scar formation in the skin (Soo et al., 2000). However, the findings in the vocal fold are puzzling because an increase in collagen synthesis should correspond with decreased fibromodulin expression. Sixty days following injury, this is not the case, suggesting a potential imbalance in the wound healing response associated with vocal fold scar formation (Thibeault, Gray et al., 2002). As for decorin, the role of fibromodulin in scar formation deserves investigation and may be a target for antifibrotic therapies.

Not surprisingly, increased levels of fibronectin have been reported in immature scar as described by Thibeault, Bless, and Gray (in press). This finding corresponds well with previous investigation regarding the role of fibronectin in dermal scar formation (Kirscher & Hendrix, 1983). Fibronectin has been implicated in the formation of adhesion proteins required for epidermal cell/basement membrane attachment and epidermal cell migration. Therefore, Thibeault, Bless, and Gray (in press) hypothesized that increased fibronectin following vocal

fold injury may encourage epithelialization. The authors, however, did not mention these findings in the context of vocal nodules. As mentioned previously, vocal nodules are characterized by a disruption of the basement membrane zone. Therefore, it is a logical assumption that increased fibronectin depositions at the site of nodules are a component of the reparative process associated with re-attachment of the epidermal/basement membrane zone complex.

Rousseau et al (2003) described the development of vocal fold scar six months following surgical injury. As early as two months following surgical removal of the epithelium and lamina propria in a canine model, the authors reported no significant difference in collagen density between injured and normal vocal folds. Thick bundles of collagen were noted with general disorganization. However, at six months following injury, collagen density was significantly increased when compared to normal (Rousseau et al., 2003). These findings correspond with collagen density data for scar in the skin.

Although animal models can be used to follow the sequence of wound healing events that accompany vocal fold injury, human studies will be required for subsequent translation of findings to clinical management choices. For obvious reasons, human studies cannot currently involve a precise examination of vocal fold micro-architectural changes during wound healing. Less invasive techniques for monitoring wound healing hold the best promise for human studies. As discussed previously, recent studies have attempted to monitor vocal fold wound healing through the biochemical analysis of secretions collected from the site of injury. Branski and colleagues (2003) reported differential temporal expression of IL-1β and PGE-2 following surgical injury in a rabbit model. Maximal expression of IL-1β occurred one day following injury with resolution to baseline levels by seven days following injury. In contrast, PGE-2 did

not significantly increase immediately following injury. Maximal PGE-2 expression occurred seven days following injury and resolution to pre-injury levels was not achieved by 21 days after injury (the endpoint of the study). This type of investigation may yield insight into the wound healing process and provide therapeutic targets to minimize scar formation.

3.4. Chemical/Thermal Injury

Given their role as gatekeepers to the airway, the vocal folds are exposed to a myriad of irritants. Clinically, the most common etiologies within this category associated with voice complaints are cigarette smoke, allergies, inhaler treatments, and laryngopharyngeal reflux. However, several reports in the laryngology literature describe unique thermal injuries associated with the aspiration of hot liquid. In addition, there are emerging reports of upper airway burns from cocaine pipe screen ingestion. Obviously, vocal function is not the primary target of therapy in severe cases that may yield impaired respiratory function. More commonly, patients' voice complaints appear to be related to exposure to airborne irritants such as cigarette smoke.

Reinke's edema (RE), a common clinical entity, is associated with prolonged exposure to the irritants in cigarette smoke. Clinically, patients present with vast, diffuse edema and erythema of the true vocal folds. RE is associated with hemorrhage, increased fibrin deposition, edematous lakes, and thickening of the basement membrane zone. Reinke's edema likely represents an arrest in the normal reparative process due to prolonged inflammatory exposures (Dikkers & Nikkels, 1995).

Clinical evidence suggests that increased vocal fold irritation and inflammation are found in association with prolonged use of inhaled β-agonists or steroids for the treatment of restrictive pulmonary disease. However, few empirical data exist suggesting a connection between inhaler

use and dysphonia, and the mechanism of irritation in such cases has not been described. It is unclear whether associated laryngitis is due to the effects of the medicine or the carrier.

In addition, case reports of dysphonia associated with chemical fume exposure have been described. Dysphonia has been connected to prolonged exposure to Freon, formaldehyde, and mercury among other substances. Even the performing arts community is not immune to exposure. Richter and colleagues (2002) identified five potentially toxic substances that may affect professional opera singers. These include formaldehyde, cobalt, aromatic diisocyanates, and other agents. It is unclear why certain individuals may be more susceptible to reactions from airborne irritants than others. One hypothesis may be that individual thresholds vary for elicitation of a wound healing response. This notion is completely hypothetical at this point, but warrants further investigation.

The most researched source of chemical injury to the vocal folds is laryngopharyngeal reflux (LPR). Approximately 50% of patients with laryngeal and voice disorders have pH-documented LPR (Koufman, 1991; Koufman, Amin, & Panetti, 2000). Clinically, this population presents with diverse symptomology and endoscopic findings. Most commonly, patients present with edema of the true vocal folds that ranges from mild to severe polypoid degeneration, pseudocyst, erythema, subglottic edema, and posterior commissure hypertrophy (Belafsky, 2003). Emerging data suggest that laryngeal epithelial defenses to refluxate are substantially different from those in the esophagus. Specifically, several factors may determine the detrimental effects of acid and pepsin in the larynx including decreased expression of CAIII protein and down regulation in the expression of the secreted mucin gene MUC5AC in the context of LPR (Johnston et al., 2003).

4. Wound Healing Therapy in the Vocal Folds

Phonation is crucial for oral communication and proper vocal fold function is a requisite of phonation. Therefore, the clinician interested in addressing a phonation deficit associated with vocal fold trauma must primarily address the structural issues associated with vocal fold injury, or attempt to control the vocal fold wound healing process. To date, no studies or methods have demonstrated a dramatically effective method of controlling vocal fold wound healing.

Historically, rest was the primary treatment prescribed for many voice problems. A recent study has partially validated that approach. The authors reported more rapid re-establishment of the basement membrane zone in a canine model of surgical injury following a voice rest condition when compared to a voice use condition (Cho, Kim, Lee et al., 2000). However, in humans, poor patient compliance along with the social ramifications associated with voice rest limit it as a therapeutic option in many cases. Moreover, ongoing studies are addressing the question of whether some forms of voice use can actually improve outcomes. A speculation is that some forms of vocalization under the direction of a speech-language pathologist may allow for the requisite communication while facilitating optimal tissue mechanics.

Research in other tissue domains supports this notion and will be discussed at length in the following pages. Briefly, for example, periodontal ligament cells exposed to IL-1β and low magnitude cyclic tensile strain demonstrate significant inhibition of PGE-2 as compared to cells exposed to IL-1β alone. In addition, low magnitude cyclic tensile strain in the context of IL-1β-induced inflammation inhibits transcription of several other pro-inflammatory genes such as IL-1, IL-6, IL-8, MMP-1, MMP-3, and MMP-9, in other cell types (Agarwal et al., 2003; Long, Buckley, Liu, Kapur, & Agarwal, 2002; Long, Hu, Piesco, Buckley, & Agarwal, 2001). In addition to the acute, anti-inflammatory actions, mechanical stress may also impact long-term tissue organization following injury. Fibroblast exposure to IL-1β, stimulating an inflammatory

phenotype, decreases proteoglycan synthesis *in vitro* (Qwarnstrom et al., 1993). However, cyclic tensile strain suppresses the catabolic effects of IL-1β and TNF-α with regard to proteoglycan synthesis in a variety of cell types (Agarwal, Long, Gassner, Piesco, & Buckley, 2001; R. J. Gassner, Buckley, Studer, Evans, & Agarwal, 2000; Long, Gassner, & Agarwal, 2001; Xu, Buckley, Evans, & Agarwal, 2000). The potential implications of these findings are that there may be a substantial role for some types of vocal fold "exercise" in treating patients with acute vocal fold injury not only to enhance resolution of inflammation, but also to guide extracellular matrix synthesis towards reduced scar formation. Because this possibility is central to the present investigation, it is discussed at length shortly.

Although voice therapy may be able to influence the wound healing process, therapy still relies heavily on patient compliance. A conceptually alternative approach to phonatory exercise is to chemically inhibit the inflammatory phase of acute vocal fold injury. A long-standing technique involves the use of steroids. Injected corticosteroids are commonly prescribed to reduce vocal fold inflammation associated with prolonged phonotrauma in high caliber voice users whose career may be limited by the presence of vocal fold edema. A 2001 case report found marked improvement in vocal function following a 6-day course of oral methyl prednisolone in a male professional singer (Watts, Clark, & Early, 2001). In addition, steroids have been employed as a valid treatment approach for Reinke's edema, a chronic inflammatory condition of the vocal folds due to prolonged mucosal irritation typically associated with smoking. Several investigators have shown a marked reduction in vocal fold inflammation following either steroid injection into the lamina propria or even topical treatment with inhalation aerosol (Moesgaard & Hojslet, 1987; Tateya et al., 2003a). There may also be some utility for steroid injection in the presence of established vocal fold lesions. In one study, 27

patients underwent endoscopic injection of triamcinolone acetonide to the site of bilateral vocal fold nodules. Post-procedure examination (1-3 weeks) revealed complete nodule resolution in 17 patients and marked decrease in nodule size in the remaining 10 (Tateya et al., 2003b). It appears that therapy targeting the inflammatory phase following injury may hold much promise for improved tissue function in the long-term.

However, steroid use, particularly in the pediatric population, remains controversial. As a result, various soluble mediators of wound healing are currently under investigation for their potential for pharmaceutical vocal fold therapy. A factor of interest is hepatocyte growth factor (HGF), a powerful anti-fibrotic agent that modulates collagen formation and TGF-β expression (Bataller & Brenner, 2001; Matsumoto & Nakamura, 1997). Investigators have characterized the therapeutic potential of HGF *in vivo* following acute surgical injury to the vocal folds in a rabbit model. Histological investigation revealed improved wound healing associated with decreased fibrosis. Rheological assessment revealed that HGF treatment decreased vocal fold stiffness, improved mucosal wave propagation, phonation threshold pressure, vocal efficiency, and glottal closure (S. Hirano et al., 2004). These findings are thought to be due to the anti-fibrotic effects of HGF on vocal fold fibroblast activity. HGF has been shown to increase hyaluronic acid synthesis and decrease collagen type I synthesis in vocal fold fibroblasts *in vitro* (S. Hirano, D. Bless, D. Heisey, & C. Ford, 2003; S. Hirano, D. M. Bless, D. Heisey, & C. N. Ford, 2003; S. Hirano, Bless, Massey, Hartig, & Ford, 2003).

Mitomycin-C, a chemotherapeutic agent, has also been used to enhance vocal fold wound healing. In addition to chemotherapeutic indications, mitomycin-C has also been shown to limit fibroblast activity and fibrosis associated with tracheal injury. In one study, topical application to the vocal folds following surgical limited fibroblast proliferation within the wound area and

limited the connective tissue response following injury (Garrett, Soto, Riddick, Billante, & Reinisch, 2001). However, therefore, although theoretically sound, the use of mytomycin-C does not appear to improve wound healing outcomes in the vocal folds.

In cases of established vocal fold scar, several surgical techniques rely primarily on implantation of biomaterials. Two results may follow. First, vocal fold pliability and compliance can be altered. Second, vocal fold volume can be increased to improve vocal fold closure and phonatory efficiency. Rosen (2000) and others have suggested that vocal fold scar is one of the most challenging voice problems clinicians face. Implatation does not directly augment the wound healing process, and moreover, implantation yields a localized vocal fold injury, and elicits the inherent immune response to the injected material. For example, the foreign body response to Teflon injection into the vocal fold has been well documented (Lewy & Millet, 1978). However, the use of autologous fat appears to elicit a minimal inflammatory response, little epithelial reaction, and minimal unexpected fibrosis (Duprat Ade, Costa, Lancelotti, Ribeiro de Almeida, & Caron, 2004). Augmentation materials should not only attempt to restore the biomechanical integrity of scarred vocal folds, but also elicit a limited immune response to minimize the alteration of the long term function of the vocal folds.

5. Exercise and Wound Healing

Relevant to the current study, emerging evidence suggests that exercise in the broad sense-or tissue motion more narrowly- can alter the timing and the ultimate outcome of the wound healing response in numerous tissue. The relative chronicity of the wound may dictate the type of exercise or mobilization necessary to elicit the most beneficial outcomes. Exercise, in the form of prolonged tension, yields biomechanically superior tissue in the presence of established fibrosis. In contrast, repetitive or cyclical movements following acute injury and inflammation appear to encourage optimal recovery in that phase. In addition, burns represent a

unique type of wound in which pressure treatment optimizes outcomes. The current series of experiments targets the role of mobilization in the context of acute inflammation. Therefore, discussion regarding exercise and wound healing will primarily revolve around the acute inflammatory response.

According to Webster's Dictionary, exercise means "to put into action for the purpose of training or developing…" For the purpose of the current review, "exercise" will encompass all facets of movement, including both active and passive motion, as well as the application of pressure or tension to the target region. The effects of exercise on wound healing have been investigated in various tissues including joints, cartilage, and tendon. However, little is known about the effects of exercise on the vocal mechanism. The review that follows describes the effects of exercise, across systems, as well as potential application of findings to the vocal folds.

It should be noted at the outset that some therapeutic exercise techniques have no applicability to the vocal folds. For example, manual therapy or soft tissue mobilization (transverse friction, myofascial release, etc.) used in physical therapy practice is at present untenable for the vocal folds. Historically, these techniques were thought to break adhesions/scar and create biomechanically improved tissue. However, current thought suggests that adhesions are not disturbed by manual therapy. Instead, the application of tension to the scar facilitates novel matrix synthesis (Threlkeld, 1992). Regardless, manual therapy in the vocal folds does not appear feasible with present technology. In addition, passive movement therapy has been employed by physical therapists for decades despite relatively few efficacy data (Di Fabio, 1992). Again, this technique has little applicability to the vocal folds. A review of these techniques will therefore be omitted from the current document.

In addition, although not relevant to the current study, there is emerging evidence suggesting a role of mobilization in the context of more chronic scar. As scar continues to perplex clinicians, a brief review of recent findings may stimulate future investigation. Arem and Madden (1976) were the first to describe scar elongation following low-load prolonged stress (LLPS) as compared to the persistence of short, unyielding scars that were not subjected to stress. Several studies have since attempted to determine the optimal duration of LLPS in the form of stretch or splinting to yield the best outcomes. Brand (1985) suggested that holding the tissue in a moderately lengthened position for a "significant time" should induce scar remodeling. Although "significant time" is not specific, it has spawned investigation to determine the duration of tension that yields optimal outcomes. This time is referred to as total end range time (TERT). It appears that end range must be maintained for at least 6 to 12 hours per day in order to obtain maximal tissue improvement (Glasgow, Wilton, & Tooth, 2003; Prosser, 1996). Specifically, Prosser (1996) reported a mean TERT of 10 hours yielded superior results compared to less time spent at end range. These data suggest the potential for the application of such techniques to the vocal folds through the use of increased pitch or prolonged vocal fold elongation exercises. However, the time required for optimal outcomes appears to be the major impediment to such therapeutic strategies relative to the vocal folds.

5.1. Exercise and Acute Injury/Inflammation

Historically, immobilization was the primary treatment modality for acute orthopedic injury. However, recent findings refute this practice. Emerging *in vitro* data suggest a positive role for cyclic mechanical stress in the context of acute inflammation. Specifically, an *in vitro* model of inflammation and the application of repetitive strain has determined the specific mechanisms associated with exercise-induced resolution of inflammation. This model involves

the application of cyclic tensile strain (CTS) to cell monolayers. In general, low-magnitude cyclic tensile strain attenuates the inflammatory response (Deschner, Hofman, Piesco, & Agarwal, 2003). The anti-inflammatory effects support a role of tissue mobilization in the acute stage following injury. These findings represent a paradigm shift, refuting the practice of immobilization following injury. However, the translation of the *in vitro* environment to the human condition is tenuous. However, the data suggest a positive role for mobilization at the cellular level. Indeed, the prescription of voice rest to treat acute vocal fold inflammation conflicts with these findings and may be counterproductive to improved vocal function.

More specifically, numerous anti-inflammatory actions associated with CTS have been described in various cell types including chondrocytes and periodontal ligament cells. Periodontal ligament cells exposed to IL-1β and low magnitude cyclic tensile strain demonstrated significant inhibition of PGE-2 as compared to cells exposed to IL-1β alone. Inhibition of PGE-2 is likely reflective of reduced inflammation and pain (Dionne, Khan, & Gordon, 2001). This hypothesis may explain the clinically relevant reduction in pain associated with tissue movement in inflammatory diseases such as osteoarthritis In addition, low magnitude cyclic tensile strain in the context of IL-1β induced inflammation inhibited transcription of several other pro-inflammatory genes such as IL-1, IL-6, IL-8, MMP-1, MMP-3, and MMP-9, in periodontal ligament cells (Long et al., 2002; Long, Hu et al., 2001). Cyclic tensile strain inhibited the IL-1β signal transduction pathway upstream of mRNA induction (Agarwal et al., 2003). Cyclic tensile strain alone (without IL-1β induced inflammation) does not stimulate pro- or anti-inflammatory actions (Long et al., 2002).

In addition to acute anti-inflammatory actions, CTS may impact long-term tissue organization following injury. Fibroblast exposure to IL-1β, stimulating an inflammatory

phenotype, decreases proteoglycan synthesis *in vitro* (Qwarnstrom et al., 1993). However, cyclic tensile strain suppresses the catabolic effects of IL-1β and TNF-α with regard to proteoglycan synthesis in a variety of cell types (Figure 2) (Agarwal et al., 2001; R. J. Gassner et al., 2000; Long, Gassner et al., 2001; Xu et al., 2000).

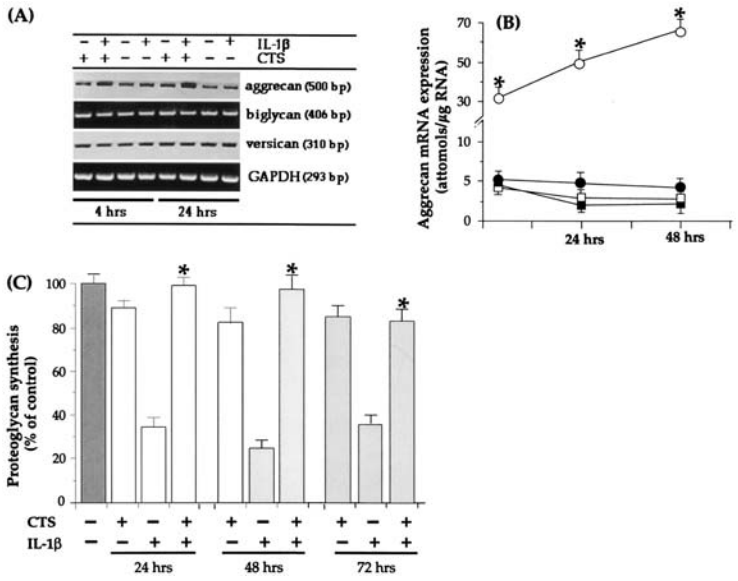

Figure 2. IL-1β and CTS affect proteoglycan synthesis

CTS negates the IL-1β-induced reduction in proteoglycan synthesis in chondrocytes (Xu et al., 2000). Copyright permission courtesy of the Amercian Association of Immunologists, Inc.

Furthermore, *in vitro* investigation of sesamoid bone cartilage from the metacarpophalangeal joints of calves subjected to mechanical loading (0.2 MPa at 0.2 Hz) for one week resulted in increased decorin synthesis when compared to cells not subjected to loading. Outside the context of inflammation or injury, the interaction between collagen and newly synthesized decorin may aid in the adaptation of articular cartilage to increased loading and has significant biomechanical implications (N. Visser, vankampen, Dekoning, & Vanderkost, 1994). Decreased decorin content has been implicated in vocal fold scar making these findings particularly relevant to that domain.

The specific pathway associated with the positive affects of CTS has been identified. Specifically, the NF-κB signal transduction pathway is the central pathway associated with the effects of tensile strain (Agarwal et al., 2003). Low-magnitude cyclic tensile strain suppresses the dissociation of nuclear factor (NF)-κB from cytosolic complexes with inhibitor(I)-κB, and therefore, prevents NF-κB's ability to enter the nucleus and trigger inflammatory actions (Agarwal et al., 2003; Ghosh & Karin, 2002; Karin & Lin, 2002). The signals induced by IL-1β and TNF-α are transmitted to the nucleus through activation of a series of kinases that lead to phosphorylation, ubiquination, and degradation of I-κB. NF-κB then translocates to the nucleus (following release from I-κB) where it binds to several pro-inflammatory genes to initiate mRNA transcription (Karin & Lin, 2002). NF-κB is important for the initiation of the inflammatory response and cell protection from apoptotic stimuli (Karin & Lin, 2002). Furthermore, NF-κB has been implicated in response to infection with enteric pathogens as well as in response to inflammatory bowel disease and diverticulitis (Rogler et al., 1998; Schreiber, Nikolaus, & Hampe, 1998).

Low magnitude tensile strain also suppresses IL-1β-dependent intracellular signaling (Agarwal et al., 2003). Because it has been shown that cyclic tensile strain does not interfere with IL-1β receptors, the specific targets of anti-inflammatory signaling may lie between the IL-1 receptor complex and I-κBβ degradation (Agarwal et al., 2003; Long, Hu et al., 2001). The specific mechanoreceptors associated with the signal transduction response to low-magnitude cyclic tensile strain remains unknown (Agarwal et al., 2003). In addition, other anti-inflammatory cytokines such as IL-10 have been shown to utilize a different pathway (Jak-STAT) to inhibit pro-inflammatory signals. This finding raises an interesting question as to why

the application of low-magnitude cyclic tensile strain directly inhibits the NF-κB pathway (Agarwal et al., 2003; Donnelly, Dickensheets, & Finbloom, 1999; Imada & Leonard, 2000)

Magnitude appears to be a critical variable to ensure the anabolic effects of CTS. High magnitudes of cyclic tensile strain applied to cell monolayers have been shown to actually induce pro-inflammatory actions (i.e., marked up-regulation of PGE-2). In addition, high-magnitude cyclic tensile strain stimulated IL-1β, TNF-α, IL-6, MMP-1, MMP-2, MMP-3, β-integrins, and plasminogen activator, which all participate in immune system activation and/or the induction of matrix degradation (Agarwal, 2001; R. Gassner et al., 1999; Long et al., 2002; Long, Gassner et al., 2001; Long, Hu et al., 2001; Yamaguchi et al., 1997). High-magnitude cyclic tensile strain is proinflammatory, and when applied in combination with IL-1β, the effects are additive, stimulating the inflammatory response (Agarwal et al., 2003). Furthermore, high-magnitude cyclic tensile strain induces NF-κB nuclear transactivation (p65 and p50 heterodimers) and eventually, COX-2 mRNA expression. The application of caffeic acid phenethyl ester, a cell permeable inhibitor of NF-κB, repeals the pro-inflammatory actions associated with high-magnitude cyclic tensile strain (Agarwal et al., 2003). In both human periodontal ligament cells and endothelial cells, high magnitude cyclic tensile strain induced sustained NF-κB nuclear translocation detected for three to four hours (Agarwal et al., 2003; Du, Mills, & Sumpio, 1995). However, NF-κB response to mechanical stress in fibroblasts (of particular interest to the vocal mechanism) was detected within two minutes with maximal expression occurring at four minutes (Inoh et al., 2002).

The dose-dependent effect of strain has been confirmed in numerous studies. For example, IL-6 (a pro-inflammatory cytokine implicated in arthropathy and osteoarthritis) expression increased in human tendon fibroblasts following 15 minutes of CTS. Interestingly,

the authors proposed that the inflammatory response associated with physiotherapy may accelerate wound healing. Furthermore, IL-6 may be an important predictor for monitoring and improving therapeutic strategies (Skutek, van Griensven, Zeichen, Brauer, & Bosch, 2001; Zeichen, van Griensven, & Bosch, 2000). However, excessive loading to intact tissue can be traumatic and lead to tissue breakdown (Buckwalter & Lane, 1997; Lequesne, Dang, & Lane, 1997).

One major problem with *in vitro* studies is the lack of information about the contributions of the ECM subsequent to mechanical stress. The ECM is the substrate to which cells adhere, grow, migrate, and differentiate. Cells adhere to the ECM via specialized cell surface receptors, integrins. Integrins are a class of heterodimeric transmembrane proteins, which bind to distinct ECM ligands (Hynes, 1992). There is evidence suggesting that mechanical stimulation via the ECM may stimulate adaptive cellular responses such as remodeling of the ECM to adjust its mechanical properties to withstand continued stress (Galbraith & Sheetz, 1998; Shyy & Chien, 1997). ECM-cell communication interactions appear critical in response to mechanical stress (Mercurio & Manning, 1999; Takahashi & Berk, 1996). Future investigation should attempt to include ECM in the application of cyclic tensile strain to further characterize this relationship and its role in the cellular adaptive response to mechanical strain.

Alternatey, CTS can have negative consequences. In addition to negative consequences from high-magnitude CTS already noted, mechanical strain has been shown to inhibit closure of airway epithelial cells *in vitro* (e.g., (Savla & Waters, 1998). These findings suggest differential cellular response to cyclic strain. Therefore, strain or "exercise" may be counterproductive to expedient wound closure following *surgical* sources of injury. This finding supports clinical practice in voice disorders, where most patients are placed on a brief period of voice rest

following phonomicrosurgery. However, in the case of vocal fold injuries involving intact epithelium (inflammation, lesions, etc.), tissue movement may be critical to both the resolution of inflammation and long-term tissue remodeling. However, to date, this hypothesis has not been tested.

A limitation of *in vitro* studies is that mesenchymal cells from different tissue types behave differently with regard to collagen and proteoglycan synthesis (Murphy, Loitz, Frank, & Hart, 1994; Phillips, Bashey, & Jiminez, 1994; J. E. Scott, 1995). Several studies have investigated the role of mechanical stress on cardiac fibroblasts and procollagen synthesis. Similar to the situation in the vocal folds, the physical properties of cardiovascular tissues are critical to adequate function. Interestingly, mechanical load alone does not alter fibroblast function in cardiac tissue. However, the presence of growth factors (or serum) that stimulate procollagen gene expression enhances loading-induced procollagen synthesis (Butt & Bishop, 1997). Results suggest that mechanical loading encourages fibrosis in cardiac fibroblasts. This finding may serve as a foundation for an alternative hypothesis for the current investigation in which the primary hypothesis is that mechanical loading will limit the fibrotic processes in vocal fold fibroblasts. Specifically, CTS may induce a fibrotic phenotype that could be detrimental to functional vocal fold tissue. However, anecdotal clinical evidence and emerging data in other tissue opposes this hypothesis.

Unfortunately, there is marked lack of *in vivo* investigation into the role of exercise in the presence of injury. Most of the data are from the orthopedic literature regarding appropriate initiation of physical therapy following surgical injury. However, there is also a body of literature investigating the biomechanical and biochemical tissue alterations associated with exercise. For example, articular cartilage decorin content in beagles subjected to a long distance

running protocol (5 days/week; 40km/day for 15 weeks) was significantly increased. However, overall glycosaminoglycan content of the cartilage did not change following the running protocol. These findings provide further confirmation that decorin synthesis is stimulated by loading. Furthermore, loading-induced decorin synthesis may be independent of the synthesis of other proteoglycans (N. A. Visser et al., 1998).

In addition, exercise increased both HA and decorin content in gastrocnemius tendons (Yoon, Brooks, Kim, Terada, & Halper, 2003). However, strenuous levels of exercise decreased proteoglycan synthesis, and caused proteoglycan aggregation, which limit biomechanical efficiency in gastrocnemius tendons (Pedrini-Mille, Pedrini, Maynard, & Vailas, 1988). These data concur with findings from *in vitro* studies suggesting there is an optimal level of "exercise" required to elicit positive tissue alterations.

With regard to joint injury, motion therapy has been shown to induce rapid recovery of inflamed joints, as well as to attenuate the inflammation-induced reduction in proteoglycan synthesis *in vivo* (Koob, Clark, Hernandez, Thrumond, & Vogel, 1992; J. M. Williams, Moran, Thonar, & Salter, 1994). Movement was thought to increase circulation and thereby disseminate inflammatory mediators away from the site of injury (Salter, 1994; Von Schroeder, Coutts, Billings, Mai, & Aratow, 1991). Furthermore, mechanical forces associated with moderate exercise have been shown to limit tissue (cartilage) degradation and facilitate tissue repair in inflamed and post-surgical joints (Haapala et al., 1999; Kiviranta, Jurvelin, Tammi, Saamanen, & Helminen, 1987; Salter, 1996; J. M. Williams et al., 1994).

Broader outcomes associated with mobilization following acute injury have been explored. Patients with lateral epichdylalgia, or "tennis elbow," were subjected to "mobilization with movement" (Mulligan, 1995). Pain ratings improved in nearly all subjects following

treatment. Interestingly, treatment also stimulated sympathoexcitatory effects in nearly all subjects. Cutaneous sympathetic nervous system-related measures (heart rate, blood pressure, skin temperature, cutaneous blood flux, and skin conductance) improved on the affected side following therapy. This response was absent in control and placebo conditions. These data suggest there may be a multisystem response associated with movement therapy (Paungmali, O'Leary, Sowvlis, & Vicenzino, 2003). The implications of these findings appear to be poorly understood and warrant further investigation.

Early motion following anterior cruciate ligament (ACL) injury is beneficial for pain reduction, capsular contraction, and range of motion limitations. In contrast, immobilization following ACL injury led to negative outcomes for the articular, ligamentous, and musculature structures surrounding the knee (Beynnon & Johnson, 1996). In addition, a 1992 study reported that 91% of patients who underwent ACL reconstruction regained full range of motion following postoperative mobilization therapy. Although the study lacked a control group, the data are encouraging.

Early mobilization is also beneficial for patients undergoing surgery for idiopathic carpal tunnel syndrome. A 1995 study found that patients placed in splints following surgery developed significantly worse outcomes when compared to patients who underwent range of motion exercises on the first post-operative day. Splinted patients experienced significant delays in the return to daily activities, return to work, and in the recovery of both grip and pinch strength. In addition, increased pain and scar tenderness was reported by splinted patients during the first month following surgery (Cook, Szabo, Birkholz, & King, 1995). These data further suggest a role for the initiation of motion exercises, acutely, following surgery for this condition.

However, contradictory data exist. A patellar tendon surgical model provides evidence against postoperative mobilization. Following resection of the tendon, a group of rabbits were placed on a treadmill exercise program for 12 weeks. A second group was placed in immobilizing casts. A third group was sacrificed immediately following surgery for control data. After three months, structural properties of all groups were similar, suggesting little benefit from exercise. The authors hypothesized that early joint mobility produces large stresses, which lead to further damage. However, the authors also suggested less aggressive exercise or increased delay between surgery and the initiation of joint mobility to facilitate functional tissue remodeling (Kamps, Linder, DeCamp, & Haut, 1994). I*n vitro* findings suggest that magnitude of exercise appears to be the critical variable.

In contrast, negative sequelae associated with immobilization following injury have been described. Numerous enzymatic adaptations occur following prolonged immobilization, which result in reduced functional mobility. Ligaments harvested following immobilization displayed increased lysosomal hydrolase activity, which degrades glycosaminoglycans. Hydrolase concentration increased as a function of the duration of immobilization. Fibroblasts appeared to switch from an anabolic to a catabolic, degradative state during immobility. The clinical implications are that rigid plaster casts following injury may limit the functional capacity of injured tissue (Gamble, Edwards, & Max, 1984).

Immobilization during development has also been investigated. As little as one month of immobilization drastically altered the biomechanical properties of the developing medial collateral ligament (MCL) in a rabbit model. The harvested MCLs showed deficits in pre-failure and strength tests. The biomechanical deficits were amplified with increased duration of immobility. Therefore, knee mobility is essential to normal maturation of the MCL (Walsh,

Frank, Shrive, & Hart, 1993). In addition, loading is critical to the establishment of functional ligamentous tissue. This response is compromised by immobilization (Thornton, Shrive, & Frank, 2003). The potential homology between these findings and the vocal mechanism will be discussed shortly.

6. Voice Therapy and Wound Healing

Principles of wound healing have been largely overlooked in the development of voice therapy programs. This oversight is unfortunate as the goal of many programs is improved tissue health. As noted previously, voice rest has been a primary treatment for many voice problems. A corollary is that voice rest may be homologous to immobilization in other connective tissue (i.e., ligament, tendon, meniscus, etc.). As described previously, there is a significant body of literature suggesting potentially detrimental effects of immobilization. Therefore, mobilization may be a critical component of rehabilitation following acute injury. In fact, one treatment program, Lessac-Madsen Resonant Voice Therapy (Verdolini, 2000) emphasizes systemic vocal exercise in mucosal injuries associated with phonotrauma, specifically for the purpose of enhancing wound healing

In addition, the overwhelming evidence suggesting significant value for CTS in the context of inflammation in other tissue provides further justification for "exercise" in the context of acute vocal fold injury. Based on other data, “exercise” may not only limit the inflammatory response to vocal fold injury; it may stimulate superior extracellular matrix synthesis following injury. Specifically, exercise likely increases proteoglycan synthesis. This possibility is particularly interesting as decreased decorin content has been implicated in vocal fold scar. Vocal exercise in the acute period following injury may improve long-term tissue structure. Therefore, immobilization or voice rest may be potentially harmful to the injured tissue. These observations are interesting and should be considered in the development of therapy programs.

However, the type of injury may play a significant role in the use of mobilization following vocal fold injury. As discussed previously, low magnitude cyclic tensile strain inhibits NF-κB, resulting in decreased pro-inflammatory gene transcription *in vitro*. However, NF-κB is critical to epithelial reconstitution in intestinal epithelial cell monolayers (Egan et al., 2003). This finding poses an interesting question regarding the utility of vocal exercise following injury involving an epithelial deficit, as occurs with surgical injury. In that case, vocal exercise may impede epithelial closure, providing scientific rationale for the findings reported by Cho and colleagues (2000) suggesting a benefit of voice rest post-surgically. These questions must be addressed in future studies beyond the scope of the present investigation.

Relevant to the present studies, although limitations abound, there appears to be sufficient information to question traditional clinical thinking in voice management. At the very least, biologic function should be considered in the development of voice therapy programs. This domain is particularly ripe for future research. Theoretically, there appears to be a role for phonation (of some type) in acute inflammation, as well as in vocal fold scar.

Verdolini (2000) suggested that resonant voice therapy or more specifically, Lessac-Madsen Resonant Voice Therapy, may aid in the resolution of vocal fold inflammation associated with phonotrauma. A case-report of a high level soprano (then touring with a traveling company) having long-standing voice difficulties associated with diffuse vocal fold edema was described. Following numerous doctor visits and various voice treatments, the patient spent approximately one hour performing resonant voice exercises. Resonant voice is characterized by large-amplitude, low-impact stress vocal fold oscillations that optimize the ration of voice output (dB) to vocal fold impact stress (D. A. Berry et al., 2001; Verdolini et al., 1998). Within 10 minutes of the initiation of resonant voice exercise, the patient reported that

her voice became clearer, with improved vocalic voice onset times and improved upper range. The patient was sent home with instructions to repeat the exercises hourly for a few minutes, 8 times a day. She was followed in the clinic intermittently over the next several days, and then sub-acutely 27 days post initial presentation. At that point, she reported that her voice was vastly improved both on- and off-stage. Her larynx appeared to have near full recovery with minimal evidence of phonotrauma (Verdolini, 2000).

In addition, other voice therapy programs such as vocal function exercises, resonant voice therapy exercises, and confidential voice therapy may all provide enhanced resolution of inflammation associated with vocal fold oscillation. Most treatment programs attempt to minimize vocal fold impact stress which may yield additional injury. However, it is unclear whether specific exercises yield improved outcomes over regular voice use. This hypothesis is currently being investigated by Verdolini and colleagues in a separate series.

The magnitude of strain may correspond to amplitude of vocal fold oscillation. If this is the case, the magnitude or amplitude of vocal fold oscillation that provides optimal anti-inflammatory actions must be determined. For example, confidential voice therapy, characterized by low-amplitude oscillations with low impact stress, may be the optimal vocal exercise for the resolution of inflammation due to the relatively low magnitude (amplitude) of strain. However, vocal fold fibroblasts may require higher levels of strain to produce anti-inflammatory actions due to the extreme biophysical demands associated with phonation. If this is the case, resonant voice therapy exercises (high-amplitude, low-impact stress oscillations) may yield optimal outcomes. Regardless, the biological ramifications of the prescribed tasks in voice therapy must be considered and warrant significant future research.

7. Summary and Hypotheses

Wound healing in the vocal folds is imperfect yielding fibrosis and resultant dysphonia. Data from other systems suggest that this outcome may be modulated through some type of tissue mobilization. However, the unique structure and function of the vocal folds limits generalization of findings to the vocal mechanism. The current study addressed that gap by employing an *in vitro* model of cyclic tensile strain to assess modulations in the inflammatory response in vocal fold fibroblasts. In addition, ECM synthesis, specifically collagen, fibronectin, decorin, and HA was assessed in response to IL-1β and cyclic tensile strain. The implications of these findings are not only critical in the context of acute phonotrauma (edema), but also relative to long-term tissue remodeling following vocal fold injury.

The general experimental hypotheses were:

1) IL-1β will induce an inflammatory cascade, as reported in other cell types, in cultured vocal fold fibroblasts *in vitro.*

2) Cyclic tensile strain (CTS) will limit the IL-1β-induced inflammatory cascade.

3) IL-1β will alter extracellular matrix synthesis, and specifically decrease decorin synthesis in human vocal fold fibroblasts.

4) CTS will limit the inflammation-induced alteration of extracellular matrix synthesis.

To address these hypotheses, a well-established *in vitro* system was used to examine the effects of mechanical strain on vocal fold fibroblasts in the context of IL-1β-induced inflammation. Although current CTS technology does not precisely replicate vocal fold movement during phonation due differences in the relative speeds of oscillation, the information obtained regarding the role of perturbation as an antagonist to inflammatory actions may be conceptually applicable to voice production.

METHODS

A general description of the methods is as follows. Vocal fold fibroblast monolayers were exposed to one of four conditions: no treatment, IL-1β exposure alone, mechanical stress alone, or IL-1β exposure plus mechanical stress. Initial experiments used rabbit vocal fold fibroblasts to confirm that the model employed in previous investigations using other tissue types applied to vocal fold fibroblasts. Once the model was confirmed, human vocal fold fibroblasts were used for all subsequent experiments. The current series of experiments utilized several different methods to obtain relevant data. The methods employed for data collection as well as the specific experimental protocols are described in the following sections.

8. Attainment of Vocal Fold Fibroblasts

Given the need to compare findings to those from previous studies in other tissues, and also the inherent difficulty in obtaining normal, human vocal fold fibroblast lines, rabbit vocal fold fibroblasts were used in the initial/preliminary experiments. Rabbit vocal fold fibroblasts (RVFF) were derived from primary cultures obtained from white New Zealand Rabbits. Whole airway samples were obtained from other investigators sacrificing animals. No animals were directly sacrificed for the completion of this study.

After obtaining tissue, the posterior margin of the larynx was bisected between the arytenoids. Although small, rabbit true vocal folds are easily visualized without magnification. The true vocal folds were then palpated to determine the anatomical location of the ventricle. Upon identification of the ventricle, a scalpel was used to make a vertical incision from the ventricular space through the underlying vocal fold. Incisions were also made at the posterior margin of the true vocal fold, just anterior to the arytenoid cartilage, and at the anterior commissure, thus freeing the vocal fold. Once free, an attempt was made to separate the lamina propria (LP) from the overlying epithelium and underlying muscle. Once isolated, the LP was

minced into small pieces. The minced tissue was then placed on 6-well plates for approximately 10 minutes to allow adherence to the plate. Approximately 3ml of complete Dulbecco's Modification of Eagle's Medium (DMEM) including 10% fetal bovine serum (FBS), 1% antibiotic-antimycotic was added to each well. The plates were then left untouched for one week, with only minimal disruption to check for mycoplasmic contamination. Assuming sufficient fibroblast outgrowth, the minced tissue was then removed from the wells and the media was replaced to facilitate rapid cell proliferation. Upon appreciable proliferation, the cells were transferred to a T-75 flask for further growth. Once confluency was achieved, the cells were plated on bioflex plates and grown to approximately 80% confluence (approximately 4-5 days) at 37ºC in 5% CO_2. All RVFF cells utilized were P3 because later passage vocal fold fibroblasts have been shown to be relatively unstable with regard to extracellular matrix gene expression (Thibeault, Li, Gray, & Chen, 2002).

The main experiments utilized a single human vocal fold fibroblast cell line. Attaining normal human vocal fold cells proved to be very challenging. Several relationships were established to attempt to acquire human cell lines. First, the Pathology Department at the University of Pittsburgh Medical Center provided cadaveric tissue obtained during autopsy. In such cases, autopsies typically occured approximately 36 hours post mortem. A total of seven larynges were obtained by this means. No viable cell outgrowth was obtained from any of these specimens. This lack of cell growth was likely due to cell death associated with the prolonged duration between death and autopsy. Secondly, tissue was obtained from several patients undergoing total laryngectomy. Although hearty cell lines were established, the donors all had significant tobacco history and marked clinical manifestations of Reinke's edema. Because the current study focused on the inflammatory phenotype of vocal fold fibroblasts, those samples

were deemed inappropriate. However, the cells were frozen for future use. In addition, it was determined that pulmonary investigators at the University of Pittsburgh Medical Center regularly obtain airway tissue via warm autopsy. These investigators were willing to supply normal laryngeal tissue. However, no enrolled patients presented during the 12- month period targeted for the study.

As a last resort, two cell lines were obtained from Dr. Susan Thibeault at the University of Utah. These cells were obtained through a tissue bank service employed by Dr. Thibeault. The shipment consisted of 11 total vials (5 of one line, passage 3; 6 of a second line, passage 4). Unfortunately, the P3 line was not viable upon thawing. However, the P4 cells, although extremely slow growing, were viable. These cells were derived from a 31 year-old male with no significant tobacco or laryngeal pathology history. Cell culture was established in T25 flasks. Upon relative confluence, these cells were trypsinized and re-plated in a T75 flask. Again, upon relative confluence, these cells were split in half and eventually split into two Bioflex® plates. Therefore, the cells were considered P6. A debt of gratitude is owed to Dr. Thibeault.

9. Exposure of VFF to cyclic tensile strain (CTS) and IL-1β

VFF were seeded on collagen type 1-coated Bioflex II plates (Flexcell International, Inc., Hillsboro, NC) at an approximate amount of 5 X 10^5 cells. The confluent cell cultures (4-5 days old) were washed with phosphate buffered saline (PBS) and incubated overnight in DMEM without FBS. To provide uniform circumferential strain on the Bioflex plate membrane, the plates were placed on the Flexcell loading station (located in a 5% CO_2 incubator) connected to a Flexcell unit with a computer-assisted cyclic vacuum controller. The Flexercell Strain Unit (Flexcell International Corp, Hillsborough, NC) provided uniform radial and circumferential strain on the membranes. The plates were positioned on a loading post (25 mm diameter) such that the membrane was deformed across the post face creating uniform biaxial strain. This

method of subjecting cells to uniform equibiaxial strain is the most widely used, showing a nearly linear relationship between vacuum level and strain. The radial and circumferential strains exerted on the membrane are calculated as:

$$\textit{Circumferential strain} = \frac{2\pi(\text{change in radius})}{2\pi\ (\text{original radius})} = \frac{\text{change in radius}}{\text{original radius}} = \textit{radial strain}$$

Vocal fold fibroblasts were divided into four groups: untreated and unstressed control cells, cells treated with IL-1β alone, cells treated with CTS alone, and cells treated with both CTS and IL-1β. Treatment with IL-1β involved pipetting the appropriate amount of 2.0ng/ml recombinant human IL-1β (FCRC, Fredrick, MD) into the appropriate well. For example, to treat cells with 1ng/ml of IL-1β, 1.5µl of IL-1β was pipetted into each well (3ml of media). After pipetting, the plates were gently agitated to ensure equal distribution of IL-1β in each treated well. Vocal fold fibroblasts exhibited minimal cell deformation based on visual inspection in both treatment groups. In addition, no appreciable cell detachment or cell death was observed.

10. Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR)

Following each experiment, the plates were washed three times with PBS. VFF were subjected to RNA extraction with the Quiagen RNA extraction kit (Quiagen Inc., Santa Clara, CA) following the manufacturer's suggested protocol for animal cells. Following extraction, a biospectrophotometer (Eppendorf Biophotometer, Eppendorf AG, Hamburg, Germany) was used to quantify the amount of RNA obtained during extraction. The appropriate amount of water was added to 1µg RNA to bring the total volume up to 10µl in a 0.2ml. eppendorf tube. The 0.2ml tube was then placed in the thermocycler (Eppendorff Mastercycler Personal) for approximately 15 minutes (65ºC) to denature RNA.

During this denaturation phase, Reverse Transcriptase (RT) Mix was prepared. The quantities (per sample) and components of the RT mix were as follows:

Dithiothreitol	2.5µl
First Strand Buffer	5µl
25mM MGCl23	3µl
2.5mM deoxynucleoside-triphospate	7µl
RNase inhibitor	1µl
Moloney Murine Leukemia Virus	1µl
Oligo (dt)	0.5µl

Twenty µl of RT was added to each RNA sample. This mixture was then incubated at room temperature for 10 minutes. Following incubation, the tubes were placed back in the thermocycler to synthesize cDNA (25 minutes at 42ºC, 5 minutes at 65ºC). Upon completion, 21µl of PCR Supermix along with 2µl of both sense and antisense primers were added to 2 µl of cDNA. The samples were placed back in the thermocycler and the PCR program was run for 35 cycles (2 minutes at 94ºC, 45 seconds at 94ºC, 1 minutes at 72ºC, 10 minutes at 72ºC), and held at 4°C upon completion. Two-percent agarose gels were prepared (1g agarose, 50ml TAE Buffer, 8µl EtBr). Eight microliters of PCR product plus 2µl of loading buffer were loaded into the gel and run at 95-100V for 15-20 minutes. The primer sequences are listed below:

Rabbit

GAPDH (548 bp) sense 5'GGTGAAGGTCGGAGTCAACGG-3'

antisense 5'-GGTCATGAGTCCTTCCACGAT-3'

iNOS (394 bp) sense 5'-ATGCCAGAT GGCAGCATCAGA-3'

antisense 5'-TTTCCAGGCCCATTCTCCTGC-3'.

Human

GAPDH (548 bp) sense 5'CGACCACTTTGTCAAGCTCA-3'

antisense 5'-AGGGGTCTACATGGCAACTG-3'

iNOS (394 bp) sense 5'-CGGTGCTGTATTTCCTTACGAGCGAAGAAGG-3'

antisense 5'-GGTGCTGCTTGTTAGGAGGTCAAGTAAAGGGC-3'.

COX-II (282 bp) sense 5'-TTCAAATGAGATTGTGGGAAAATTGCT-3'

antisense 5'-AGATCATCTCTGCCTGAGTATCTT-3'.

MMP-1 (403 bp) sense 5'-CTGTTCAGGGACAGAATGTGCT-3'

antisense 5'-TTGGACTCACACCATGTGTT-3'

Collagen Type I (400 bp) sense 5'-AGGTGGAAAAGGTGAACAGG-3'

antisense 5'-ACCATCTCTGCCAGGGTTAC-3'.

Decorin (271 bp) sense 5'-CTGCTGTTGACAATGGCTCT-3'

antisence 5'-AGGTGGATGGCTGTATCTCC-3'.

Hyaluronic Acid Synthase *2* (383 bp) sense 5'-GTTCTCTGGGCCTCAGTTTC-3'

antisense 5'-GAACGAGGAGAAAGCAGGAC-3'

The housekeeping gene, GAPDH, was used to ensure equal loading of each sample of interest. GAPDH gels were run for each condition first. These gels were then subjected to densometric analysis using ImageJ Software (National Institute of Health) and a protocol obtained from Dr. Michael T. Heneka of the University of Bonn Medical Center. For each lane in the gel, the background optical density was determined by the area above and below the band of interest using the crosshair tool. These two measurements were then averaged (B_a). The band of interest was measured twice and the results were averaged (X_a). The background was then subtracted from the band density (X_a-B_a=X') to yield the adjusted band density. All GAPDH

levels were then standardized to the lowest densometric value. This value was then used to load the appropriate amount of PCR product for other primers. This densometric protocol was used for all following analyses.

11. Enzyme Linked Immunosorbent Assays (ELISA)

Supernatant solution was collected from all wells following the 24-hour experiments. The media were then subjected to analysis using commercially available kits for PGE-2 and IL-6 (R&D, Minneapolis, MN), Collagen (Chondrex Inc., Redmond, WA), and Hyaluronan (Echelon Biosciences Incorporated, Salt Lake City, UT). The manufacturer's protocol was followed for all kits.

In addition, the Griess reaction was used to determine vocal fold fibroblast secretion of nitric oxide. The protocol for the Griess reaction was obtained from Dr. David Hackam's laboratory at the University of Pittsburgh Children's Hospital (Schmidt, 1995). The Griess reagent was made by adding distilled water to 0.5gm of N-(1-Naphthyl) ethylenediamine dihydrochloride (Sigma, #9125) to make 500 ml of solution. Distilled water was then added to 5 gm of sulfanilamide (Sigma, #N-9215) and 29.5 ml of 85% H_3PO_4 to make 500 ml of solution. To prepare Griess reagent, equal volumes of the two solutions were mixed. Stock solution of 6.4mM $NaNO_2$ standard was prepared. Serial dilutions were then made ranging from 128μM to 1μM. 100 μl of culture supernatant or standard was transferred to each microwell (in duplicate). 100 μl of Griess reagent was then added to each well. The plate was then read at 550 nm. NO concentrations were extrapolated based on the standard curve.

All ELISA and Greiss data were standardized to total protein content in the culture supernatant solution. The Biochinchronic Acid Assay (BCA) was used to this end (Wiehelman, 1988). This technique was used to eliminate any variability in cell density which may alter ELISA data. First, the BCA reagent was prepared by adding 100μL of Copper (II) Sulfate to

5mL of BCA. The protein standard, bovine serum albumin, was used to make serial dilutions with distilled water as the diluent. The next step was to add 25μL of the samples and standards to a 96-well plate. To each well 200μL of the BCA reagent was added and the plate was incubated at 37ºC for 30 min. After the incubation, the plate was read in a plate reader at 562nm. All results were determined within the concentration-dependent range of the standard curve. Therefore, all results were reported as a function of total protein (for example, ng PGE-2/mg protein).

12. Preliminary Experiments

The preliminary experiments were designed to confirm that vocal fold fibroblasts behave as expected with regard to both IL-β exposure as well as CTS. Several preliminary experiments were required. Set-Up Experiments 1 and 2 used rabbit vocal fold fibroblasts. Set-Up Experiment 3 used human vocal fold fibroblasts to confirm relative homogeneity of the cell cultures.

12.1. Set-Up Experiment 1

The first experiment sought to confirm that IL-1β elicited an inflammatory response in vocal fold fibroblasts. Furthermore, the optimal concentration of IL-1β that elicited this inflammatory phenotype was determined. In this study, iNOS mRNA expression was examined as a function of the concentration of IL-1β exposure (0.1, 0.5, 1.0. 5.0, and 10.0 ng/ml) within 4 hours in a single rabbit vocal fold fibroblast line.

12.2. Set-Up Experiment 2

The goal of the second experiment was to determine the optimal magnitude of CTS that inhibits IL-1β-induced iNOS mRNA expression in a single RVFF cell line. Six-well plates were exposed to 3%, 6%, 9%, or 18% of CTS at two frequencies (0.5 and 0.05 Hz) with and without

IL-1β treatment. In addition, a single plate was left unmanipulated to serve as a control condition. The experimental set-up is shown in Figure 3. This paradigm was utilized for all subsequent experiments. iNOS RNA expression was examined as described previously.

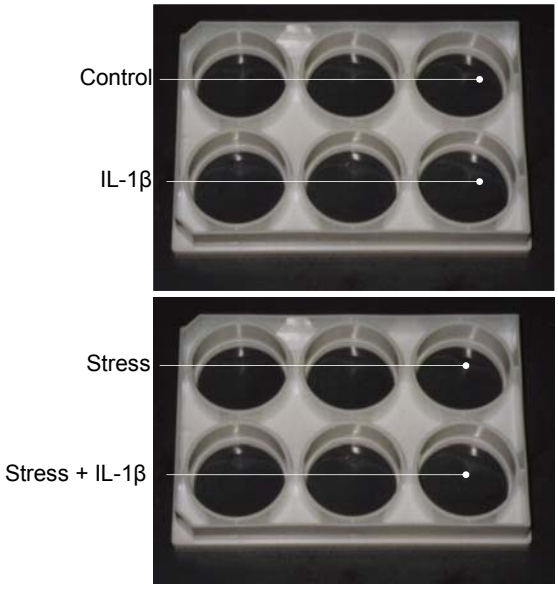

Figure 3. Paradigm used for all stress experiments

12.3. Set-Up Experiment 3

A major criticism of *in vitro* work is the potential for heterogeneity in cultures. Therefore, in order to confirm fibroblasts in culture, the following criteria were employed: 1) positive staining for vimentin, an intermediate fiber of the cytoskeleton in mesenchymal cells, 2) negative staining for vonWillebrand Factor, an endothelial cell-specific marker, and 3) collagen synthesis. Historically, vimentin staining alone was used to confirm fibroblasts in culture. Although fibroblasts stain positive for vimentin, other mesenchymal cells may also stain positive. In addition, only fibroblasts and endothelial cells produce collagen. Therefore, both enzyme-linked immunoassay for collagen as well as RT-PCR data for collagen confirms either

fibroblasts and/or endothelial cells in culture. Finally, vonWillebrand Factor, an endothelial cell-specific marker was used to differentiate between endothelial cells and fibroblasts in culture.

Immunocytochemical confirmation of fibroblasts was achieved using an aliquot of approximately 10^4 to 10^5 human vocal fold fibroblasts. In addition, human umbilical vein endothelial cells (HUVEC) were obtained from Dr. David Hackam's laboratory at the University of Pittsburgh Children's Hospital. An immunofluorescent secondary antibody was used and confocal images can be found in the results section. The following antibodies were used: 1) Monoclonal Anti-Vimentin (1:500) (Sigma Chemical Corporation, St. Louis, MO), and 2) Rabbit Anti-Human von Willebrand Factor (1:200) (DAKO Corporation, Carpinteria, CA). Ideally, RVFFs should have been subjected to similar confirmation. However, several problems were discovered. First, the endothelial cell specific marker was created in rabbit. Therefore, attempts at negative staining of RVFF were unsuccessful. In addition, gaining access to rabbit-derived endothelial cells to serve as a positive control was problematic. Therefore, only HVFFs were confirmed in culture. Given the interspecies variability noted in the current investigation, the lack of RVFF confirmation is problematic and will be discussed in later sections of the document.

The protocol utilized for all immunohistochemistry was as follows:

1) Grow cells to relative confluence on 18mm round coverslips.

2) Fix cells with 4% paraformaldehyde (20 minutes).

3) Suction paraformaldehyde.

4) Permeabilize cells with 0.1% Triton-X-100 (20 minutes).

5) Suction Triton

6) Wash three times with phosphate buffered saline (PBS).

7) Block with protein blocking agent (45 minutes).

8) Suction blocking agent.

9) Incubate with primary antibody at room temperature (1 hour).

10) Wash three times with PBS.

11) Incubate with secondary/fluorescent antibody at room temperature (45 minutes).

12) Wash three times with PBS.

13) Place coverslips in aqueous mount on slides.

13. Main Experiment 1 (Specific Aim # 1)

13.1. Purpose

The purpose was to examine the pro-inflammatory effects of interleukin-1β (IL-1β) on human vocal fold fibroblasts (HVFF), and quantify the effects of cyclic tensile strain (CTS) on IL-1β-induced pro-inflammatory responses. This Specific Aim was addressed by:

(a) Assessing the IL-1β-induced mRNA expression and protein synthesis of pro-inflammatory molecules, such as cyclooxygenase-II (COX-II), inducible nitric oxide synthase (iNOS), as well as prostaglandin E-2 (PGE-2) and nitric oxide (NO) synthesis, and (b) Assessing the effects of CTS on IL-1β-induced pro-inflammatory actions. Specifically, the effects of CTS were examined on the expression of COX-II and iNOS mRNA, as well as PGE-2 and NO synthesis.

13.2. Procedures

HVFF were exposed to optimal concentrations of IL-1β and the optimal magnitude of CTS required to inhibit IL-1β actions, as determined in Set-up Experiments 1 and 2. Main Experiments were completed in triplicate. The expression of COX-II and iNOS mRNA were

examined at 4 and 24 hours. PGE-2 and NO production were assessed in the culture supernatant at 24 hours only.

13.3. Measurement of Dependent Variables

PGE-2 (pg/mg protein) was measured in the culture supernatant solution 24 hours following treatment via enzyme-linked immunosorbent assay (ELISA) (R&D Systems, Minneapolis, MN).

NO (μM/mg protein) was measured in the culture supernatant solution 24 hours following treatment via Griess reaction (Grisham, Johnson, Gautreaux, & Berg, 1995)

iNOS (mRNA Expression) and **COX-II** (mRNA expression) were assessed via RT/PCR and densometric analysis described previously 4 and 24 hours following treatment.

14. Main Experiment 2 (Specific Aim # 2)

14.1. Purpose

One purpose was to examine the effects of interleukin-1β (IL-1β) on extracellular matrix (ECM) synthesis in HVFF, and determine whether the actions of CTS on HVFF result in the upregulation of ECM synthesis in the presence or absence of IL-1β. This goal was addressed by:

(a) Assessing extracellular matrix synthesis by HVFF in the presence of IL-1β; and

(b) Assessing the effects of CTS on extracellular matrix synthesis in the presence of IL-1β.

14.2. Procedures

HVFF were exposed to optimal concentrations of IL-1β and the optimal magnitude of CTS required to inhibit IL-1β actions, as determined in Set-Up Experiments 1 and 2. Main Experiments were completed in triplicate. The expression of collagen, hyaluronic acid synthase, decorin and fibronectin mRNA were examined at 4 and 24 hours. Collagen and HA production were assessed in the culture supernatant at 24 hours only.

14.3. Measurement of Dependent Variables

Collagen, Hyaluronic Acid Synthase (2), Decorin, and **Fibronectin** (mRNA Expression) were assessed via RT/PCR and densometric analysis described previously.

Collagen Type I (ng/mg protein) and **Hyaluronic Acid** (pg/mg protein) were measured in the culture supernatant solution 24 hours following treatment via enzyme-linked immunosorbent assay.

15. Data Analysis

The type of research described in the current series of experiments is relatively novel to the field of communication sciences. In contrast to more biological sciences, the field of communication sciences relies heavily upon statistical analysis for confirmation of results. The use of statistics in that realm is necessary given variability within and across human behaviors, which may confound conclusions regarding the applicability of the findings to the greater population. In contrast, many more basic science endeavors are limited by both the availability of experimental materials in addition to the relatively artificial *in vitro* environment. In these cases, the trend in data analysis is to observe qualitatively relevant phenomena, not attempting to insert limited data into a statistical model in which it does not fit (for example, Agarwal, 2001; Agarwal et al., 2001).

The current study investigated the differences in (a) gene expression and (b) molecule synthesis using ELISA, in a single cell line. Relative to gene expression, although the experiments were performed rigorously and the results have relevance, no quantifiable data was obtained. Furthermore, after discussing the data at length with numerous investigators, the overwhelming suggestion was to show exemplar gels from electrophoresis as a primary dependent variable (Sudha Agarwal, personal communication). Therefore, all gene expression data are presented visually. In some cases, densometric analysis was performed to yield some

quantification to the data. Although densometry provides some degree of quantification, those data are not without problems. Primarily, polymerase chain reaction yields exponential amplification of gene products. Therefore, quantifying differences that have been amplified to such a marked degree is problematic. In contrast, semi-quantitative methods exist to examine gene expression data. However, such methods were not employed in the current study due to limitations associated with both expense and availability of equipment. Regardless, even in the face of emerging quantitative technology, qualitative PCR remains common in describing gene expression results.

In contrast, ELISA presents somewhat greater possibility to analyze the data statistically due to the generation of numeric data from the standard curve. At the descriptive level, the data are presented in terms of means and standard errors across the triplicate experiments. At the inferential level, these data were subjected to one-way analyses of variance (ANOVAs) in spite of the fact that the data do not meet all of the assumptions required for this test. Although the assumption of homogeneity of variance was met in most cases, the assumption of normality was not. Clearly, experimentation on a single cell line does not represent randomly selected samples within a population. However, in the absence of an appropriate model, most literature in this realm utilizes the ANOVA (for example, Long, Hu et al., 2001). Therefore, two ANOVAs were performed in each main experiment with treatment condition the independent variable and synthesis of relevant molecules the dependent variable. The p-value for each main experiment was set at 0.05 and adjusted appropriately for two tests (0.05/2=0.025). Assuming significance of the ANOVA, post-hoc analyses were performed between the conditions using the Tukey calculation.

RESULTS

Stated simply, the series of experiments produced a step-wise progression that indicated the following findings: 1) the cells cultured from vocal folds were fibroblasts, 2) vocal fold fibroblasts responded similarly to other cell types with regard to inflammatory stimulation by IL-1β, 3) cyclic tensile strain, at low levels and for smaller durations, attenuated the inflammatory response to inflammatory stimuli, and 4) neither IL-1β nor CTS altered extracellular matrix gene expression or synthesis. Details are indicated next.

16. IL-1β stimulated iNOS expression in vocal fold fibroblasts (Set-Up Experiment 1)

As shown in Figure 4, 1.0ng/ml of IL-1β was the minimal dosage that stimulated iNOS expression in RVFFs. Concentrations lower than 1ng/ml did not elicit the inflammatory phenotype. Furthermore, beyond 1ng/ml, increased IL-1β concentrations did not further increase iNOS expression. Based on this information, 1ng/ml of IL-1β was used for all subsequent experiments.

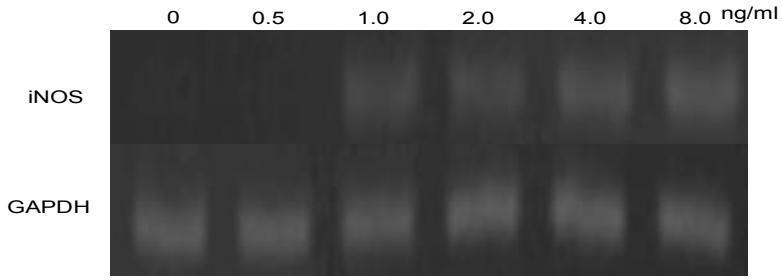

Figure 4. iNOS expression as a function of IL-1β concentration

17. Cyclic tensile strain suppressed the IL-1β-induced inflammatory phenotype in RVFF (Set-Up Experiment 2)

Following confirmation that IL-1β stimulated iNOS expression, the next step was to determine if cyclic tensile strain suppressed the inflammatory signal. Although CTS may be applied at a wide range of frequencies and magnitudes, the literature suggests that low-magnitude CTS is the most effective inflammatory suppressor. No previous investigation has determined a trend with regard to the relative effect of frequency on inflammatory suppression. Frequency is particularly relevant to the vocal mechanism. The maximal frequency permitted by the Flexcell® unit is 0.5Hz. This restriction in frequency is a potential limiting factor for generalizing findings from the current investigation. Therefore, the preliminary experiment targeted iNOS gene expression as a response of both frequency and magnitude of CTS. As shown in 5, both 0.05 and 0.5Hz suppressed iNOS expression at low magnitudes. At 0.01Hz and 3% CTS (magnitudes defined previously), approximately 90% iNOS suppression was noted when compared to cells exposed to IL-1β alone. In addition, an approximate 60% suppression of iNOS expression was noted at low magnitudes and 0.5Hz. As reported for other tissues, higher magnitude CTS does not suppress inflammation. Figure 5 contains concurring data. Based on these findings, subsequent stress experiments were conducted utilizing 3% stress at 0.5Hz (1 second stress followed by 1 second of rest).

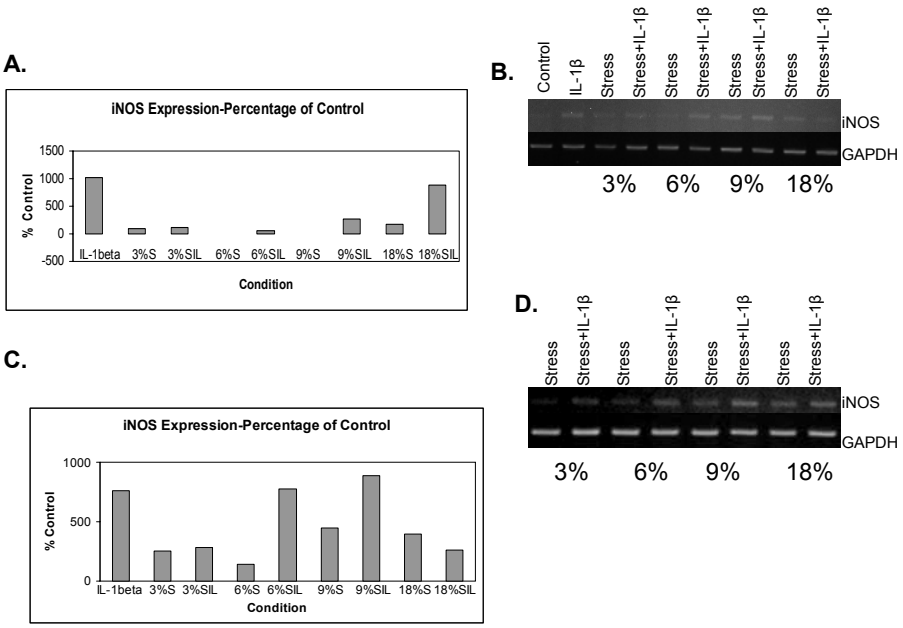

Figure 5. Results of preliminary stress experiment

Preliminary experiments showed clear attenuation of the iNOS expression in response to CTS at low magnitudes. Panels A and B show iNOS gene expression following CTS at 0.05 Hz. Panels C and D show iNOS expression in response to CTS at 0.5 Hz.

18. Fibroblasts were in culture (Set-Up Experiment 3)

As shown in Figure 6, the HVFFs supplied by Dr. Susan Thibeault were a homogenous fibroblast population. These cells stained vimentin positive, von Willebrand negative, and produced collagen.

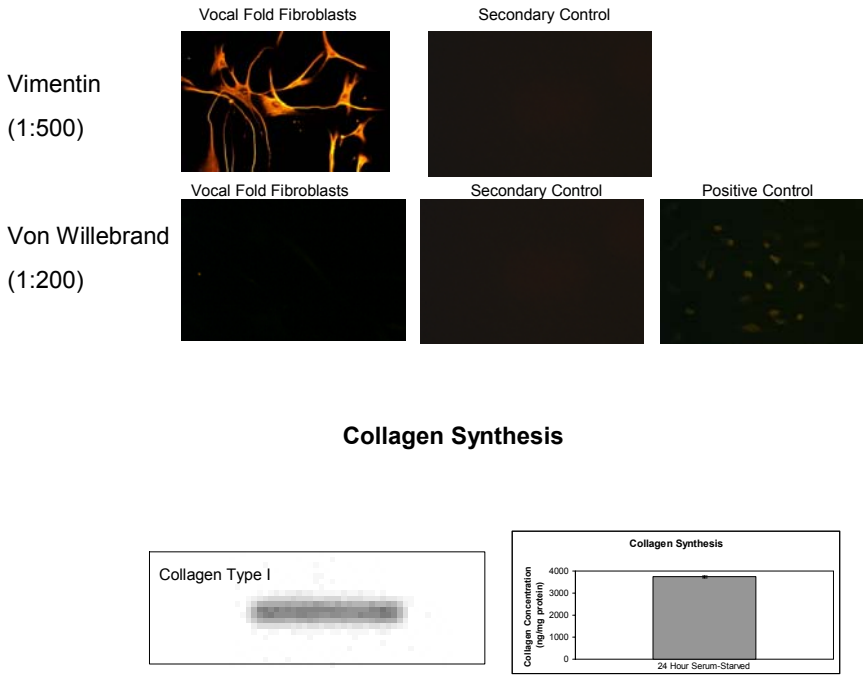

Figure 6. Confirmation of fibroblasts in culture

Vocal fold fibroblasts stained positive for vimentin, negative for von Willebrand Factor, and produced collagen (assessed via PCR and ELISA)

19. CTS suppresses the IL-1β-induced inflammatory gene induction in human vocal fold fibroblasts (Main Experiment 1)

Positive findings for the initial experiments permitted progression to the use of human vocal fold fibroblasts. Specifically, 1.0 ng/ml of IL-1β stimulated iNOS synthesis in RVFF. In addition, 3% CTS at both 0.05 and 0.5 Hz attenuated the inflammatory response with regard to iNOS expression. Based on those findings, HVFF were exposed to four conditions: Control (no treatment), IL-1β (1.0 ng/ml), CTS alone (3%, .5Hz), and CTS (3%, .5Hz) plus IL-1β for 4 and 24 hours. Initially, iNOS expression was a variable of interest. However, multiple attempts to elicit iNOS gene expression with IL-1β in human vocal fold cells lines were not fruitful. Therefore, a positive control experiment was run using MC63, a human osteoblast-like cell line. These cells have been shown to express iNOS in response to IL-1β. Figure 7 shows gel

electrophoresis for iNOS in both MC63 cells as well as HVFF. In addition, the figure shows Greiss reaction results for HVFF in a control condition and following 24-hour exposure to IL-1β. Both values fell below the lowest value of the standard curve (R^2=0.99). Therefore, both of these values may be considered zero.

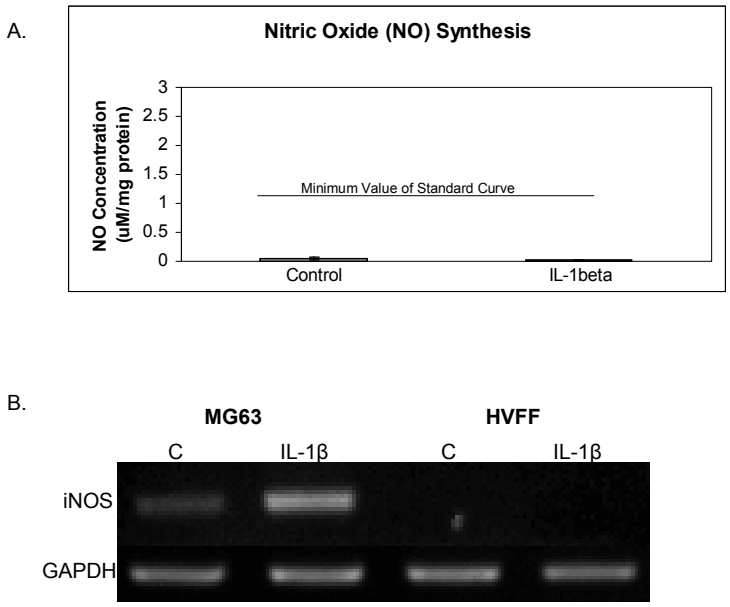

Figure 7. HVFF do not express iNOS or produce NO in response to IL-1β

Panel A shows results from Greiss Reaction in control and IL-1β stimulated human vocal fold fibroblasts. Panel B shows iNOS gene expression in a positive control cell line and HVFF in both control and IL-1β treatment conditions.

These results suggest the possibility that human vocal fold fibroblasts do not express iNOS. Although this finding warrants further investigation, another inflammatory mediator was identified as a potential target for investigation, matrix metalloprotease-1 (MMP-1). Previous investigation in Dr. Agarwal's laboratory has suggested that MMP-1 is sensitive to both IL-1β and CTS. Therefore, MMP-1 was included as a dependent variable in addition to COX-2. As

mentioned previously, all experiments were run for both 4 and 24 hours. For simplicity's sake, 4 hour and 24 hour data are presented separately.

19.1. Four-Hour Experiment

Both COX-2 and MMP-1 gene expression increased in response to IL-1β. In addition, the IL-1β-induced expression of both genes was attenuated by CTS (Figures 8 and 9). Densometric analyses showed approximately 35% and 30% suppression of COX-2 and MMP-1, respectively, in response to CTS. Although the suppression is not of the same magnitude of suppression noted in previous reports in other systems, it appears to be a qualitative reality.

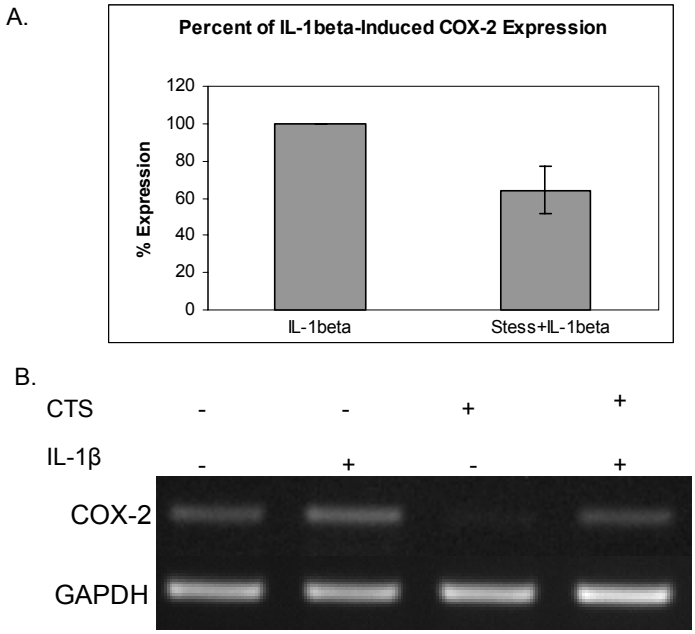

Figure 8. Four hours of CTS attenuates IL-1β-induced COX-2 expression

Panel A shows densometric analyses of COX-2 gene expression as a function of IL-1β stimulation and CTS. Panel B shows a representative gel for COX-2 expression.

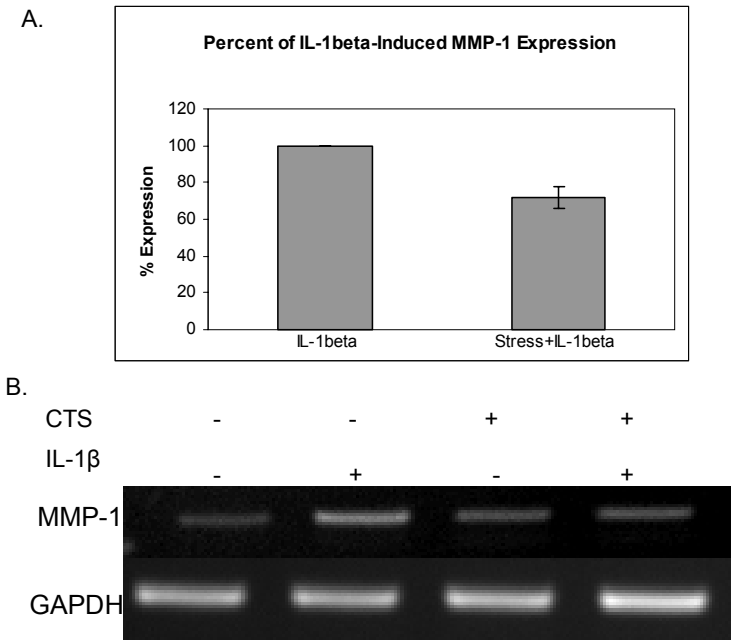

Figure 9. Four hours of CTS attenuates IL-1β-induced MMP-1 expression

Panel A shows densometric analyses of MMP-1 gene expression as a function of IL-1β stimulation and CTS. Panel B shows a representative gel for MMP-1 expression.

19.2. Twenty-Four Hour Experiment

At 24 hours, the data become a bit more variable. IL-1β stimulation of inflammatory gene expression (COX-2 and MMP-1) was less apparent that at 4 hours. In addition, the relative effects of CTS were difficult to interpret. With regard to COX-2, neither IL-1β stimulation nor CTS had a consistent effect on gene expression (Figure 10). In addition, presentation of a representative gel was inappropriate in this case due to the increased variability. However, analysis of PGE-2 synthesis suggested that stress plus IL-1β dramatically increased PGE-2 synthesis in the supernatant solution. Although the ANOVA suggested overall significance ($p<0.0001$), post hoc analysis suggested that IL-1β did not significantly increase PGE-2 synthesis ($p=0.252$). In addition, stress alone did not increase PGE-2 levels compared to

untreated cells ($p=0.970$). However, stress plus IL-1β did significantly increase PGE-2 ($p<0.0001$).

These findings were contrary to the experimental hypothesis. In order to ensure that the findings were not spurious, the supernatant solution was subjected to ELISA for interleukin-6 (IL-6), another pro-inflammatory cytokine (Figure 11). Identical to the PGE-2 data, the overall ANOVA was significant ($p=.001$). Furthermore, results from the post hoc analyses were nearly identical to those for PGE-2. IL-1β did not appear to stimulate IL-6 to a significant degree ($p=0.946$). In addition, stress alone did not induce IL-6 ($p=1.0$). However, stress plus IL-1β significantly increased IL-6 production ($p=0.018$).

In order to further confirm these findings were accurate, a third ELISA was performed (data not shown) for IL-1β. This ELISA was necessary to confirm two issues: 1) to ensure the IL-1β used in the experiment was active, and 2) to ensure equal dosages of IL-1β were applied to each treatment well. Equal dosage in the IL-1β treatment wells and the CTS plus IL-1β wells is critical to the experimental hypotheses. The results of this ELISA found no IL-1β in the control or stress-only solutions. However, concentrations of IL-1β were similar (~24pg/ml) in both the IL-1β alone and CTS plus IL-1β conditions. Therefore, the results of the IL-1β ELISA further validated the data obtained for PGE-2 and IL-6 indicating that CTS plus IL-1β appeared to have an additive effect on inflammatory mediator synthesis following 24-hours of treatment.

The MMP-1 response at 24-hours was also quite variable with some gels displaying marked suppression of the inflammatory signal and others actually amplifying the inflammatory signal (Figure 12). Again, inclusion of a representative gel was inappropriate due to this variability. Although the data for the 4-hour experiments are promising, these 24-hour experiments generate more questions than answers (Figure 13).

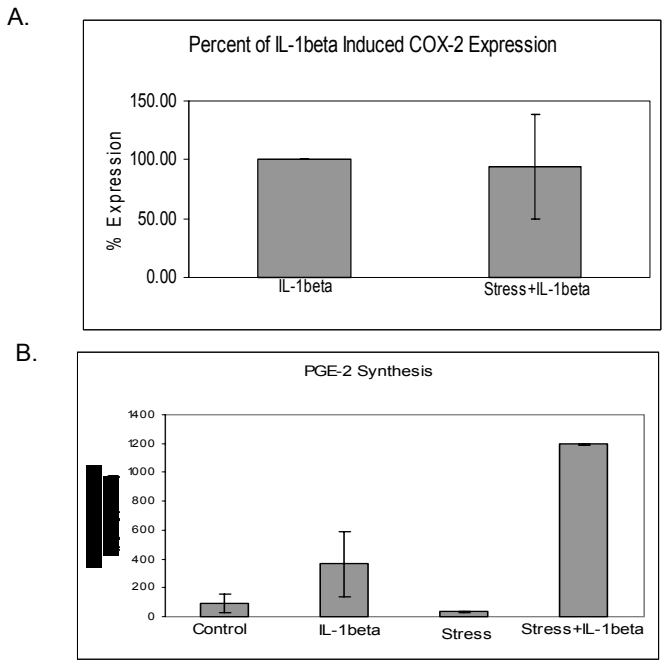

Figure 10. Twenty-four hour experiment results

Panel A shows densometric analysis of COX-2 expression as a function of IL-1β and CTS for 24 hours. Panel B shows PGE-2 synthesis as a function of the four treatment conditions over 24 hours.

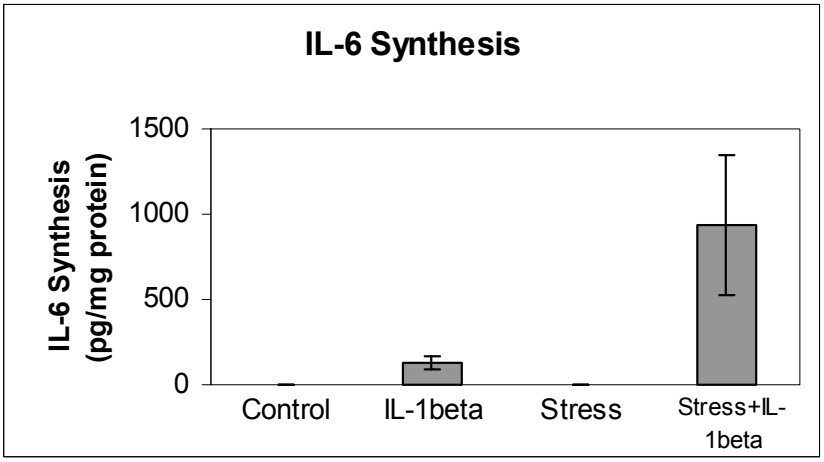

Figure 11. ELISA data for IL-6 (24 hours)

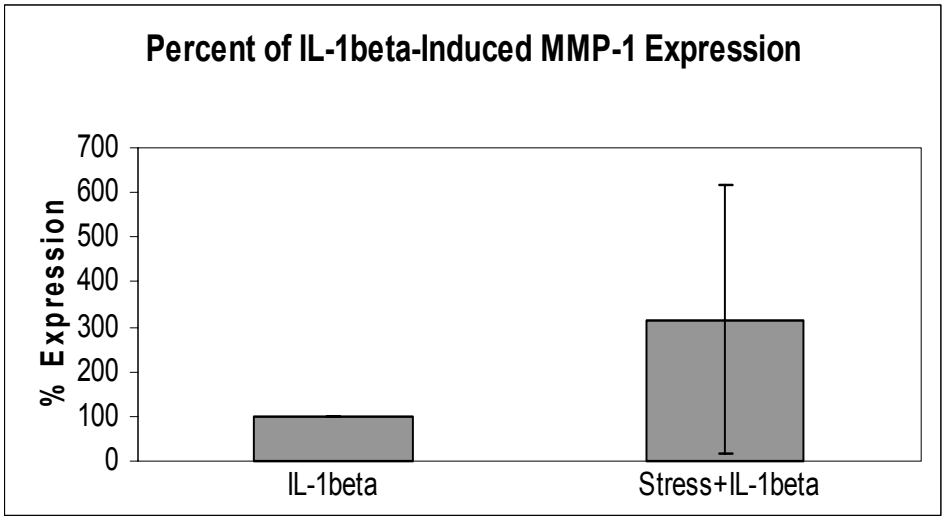

Figure 12. MMP-1 results for 24 hour experiment

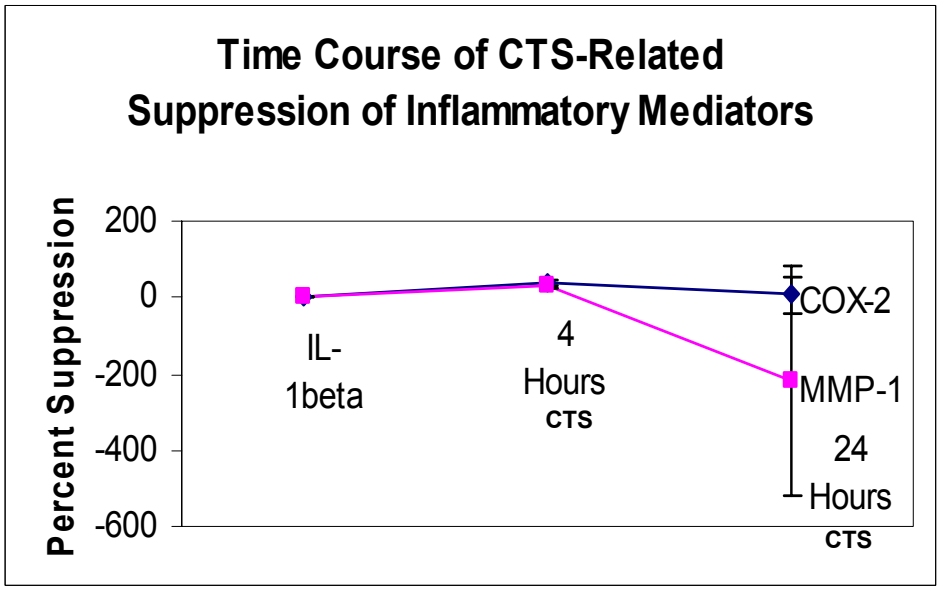

Figure 13. Temporal course of CTS-induced suppression of inflammation

20. Neither IL-1β nor CTS altered the synthetic properties of vocal fold fibroblasts (Main Experiment 2)

As described previously, IL-1β has been shown to modify the synthetic characteristics of other cells types. Most critical to the vocal fold microarchitecture are collagen, hyaluronic acid (HA), fibronectin, and decorin. The second experiment sought to determine if vocal fold fibroblasts respond similarly to inflammatory stimuli and mechanical stress. Using the paradigm described previously, HVFF were exposed to four conditions: Control (no treatment), IL-1β (1.0 ng/ml), CTS alone (3%, 0.5Hz), and CTS (3%, 0.5Hz) plus IL-1β for 4 and 24 hours. Gene expression for Collagen, HA, fibronectin, and decorin was observed. In addition, both collagen and HA synthesis were determined via enzyme-linked immunoassay of the supernatant solution following 24 hours of treatment.

20.1. Four-Hour Experiment

No differences were noted with regard to collagen, decorin, and fibronectin gene expression across the experimental conditions (Figure 14). Furthermore, primers utilized for hyaluronic acid synthase (2) were not successful in isolating gene expression data. Three isoforms of the hyaluronic acid synthase have been identified. The second isoform, HAS-2, was identified as the most common in this type of research. However, this isoform may not be relevant to vocal fold fibroblasts. The particular enzyme responsible for HA synthesis in the vocal folds may be a target for anti-fibrotic therapy as decreased HA concentration has been described in vocal fold scar.

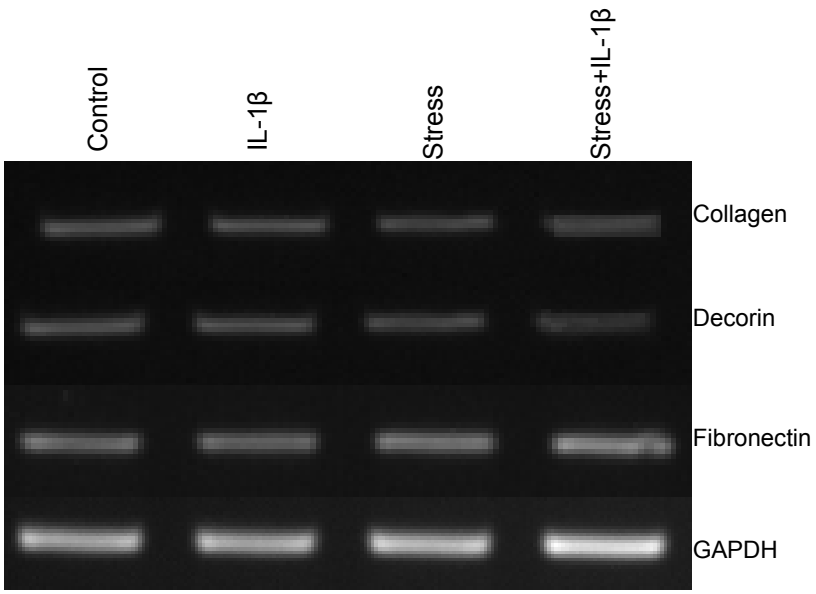

Figure 14. Four hour ECM experiment

20.2. Twenty-Four Hour Experiment

Similar to the 4-hour results, no qualitative or quantitative differences were noted with regard to either IL-1β treatment or the response to mechanical stress at the 24-hour timepoint. As shown in Figure 15, no qualitative differences in gene expression were obtained. Furthermore, ELISA data for both collagen and HA synthesis (Figure 16) revealed no significant differences ($p=0.298$ and $p=0.115$, respectively).

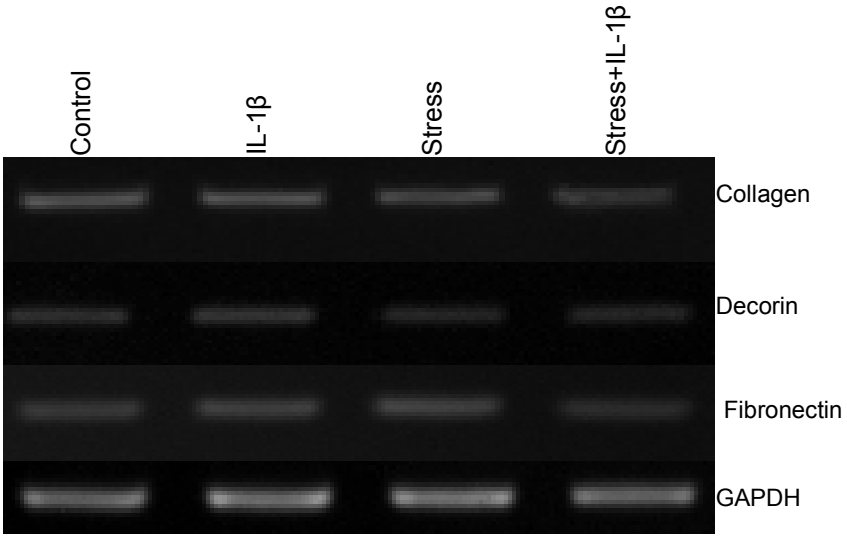

Figure 15. Twenty-four hour ECM experiment

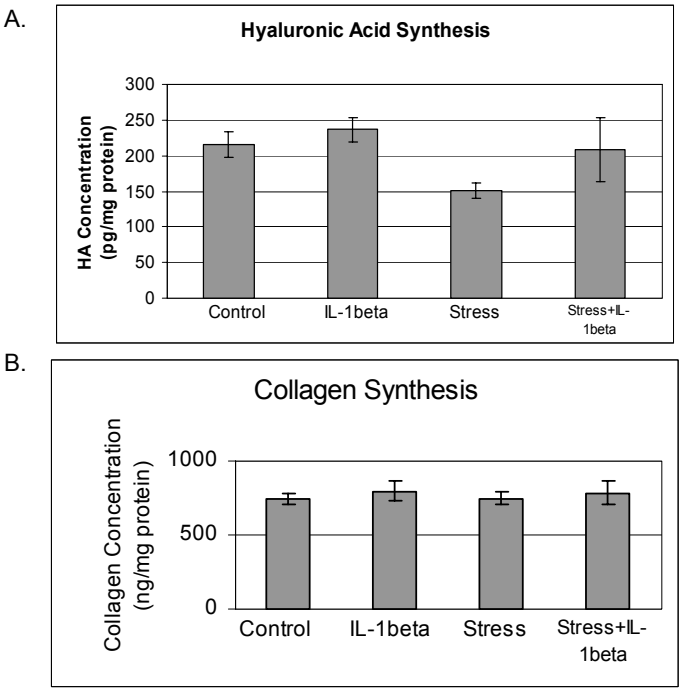

Figure 16. ECM ELISA data

DISCUSSION

The primary question addressed in the current study was whether mechanical stress alters the inflammatory response in vocal fold fibroblasts. Overall, results from the current series of experiments suggest positive findings following short-term (4-hour) stress regimens. However, data from 24-hour experiments were negative. Four hours of CTS suppressed inflammatory gene expression. Twenty-four hours of CTS did not. Results suggested that 24 hours of CTS may, in fact, have induced increased inflammatory gene expression. These findings may confirm the hypothesis that some sort of threshold of stimulation exists for mechanical signaling. Below this threshold, mechanical stress may be beneficial. However, once the threshold is surpassed, a more degradative outcome results. Data from the current study suggest that similar levels of mechanical stress may have stimulated an anabolic response in vocal fold fibroblasts in four hours. However, this same level of mechanical stress, in the longer term, may have facilitated a catabolic response. Applying this line of reasoning to other systems, little joint injury is induced in walking a short distance. However, increased injury is likely incurred if sprinting a short distance. Furthermore, although walking a short distance may not elicit injury, a durational threshold exists at which point walking becomes traumatic. For example, walking for 24-hours may induce joint inflammation. To relate this analogy to voice use, shorter durations of low intensity voice use with low impact stress does not likely evoke a massive inflammatory response. However, if low intensity voice use is prolonged an inflammatory response is likely. In contrast, a very short episode of high-intensity voice use is likely to evoke a similar response. This rationale appears to provide a complete and thorough explanation of the current findings. This topic warrants further investigation, but more basic discussion of the findings and potential implications are necessary.

With regard to the *in vitro* model, it appears that an inflammatory phenotype was stimulated in vocal fold fibroblasts. Although creating this model was not a primary goal of the current study, hopefully, the model may serve in future investigations regarding the inflammatory phenotype in vocal fold fibroblasts. IL-1β induced an inflammatory response in both human and rabbit vocal fold fibroblasts. However, the interspecies variability in the inflammatory response was interesting. The lack of iNOS expression in HVFF in contrast to RVFF warrants further investigation. Both gene expression and Greiss reaction data suggest that HVFF do not produce NO in response to IL-1β stimulation. The lack of iNOS expression has been described in several other cell types, but given the assumption of relative homology between the species, this finding is interesting. One potential source for the discrepancy may be the tissue harvest technique utilized. Perhaps either the rabbit or human cell lines used in the current investigation were contaminated with fibroblasts from either the sub- or supra-glottis. Furthermore, cell phenotypes may vary within the vocal fold lamina propria. Investigators have already identified a unique cell type with distinct synthetic properties within the macula flava of the vocal fold (Sato et al., 2001). There may also be distinct differences among cells located in different anatomic locations within the lamina propria.

Harvesting fibroblasts from the vocal fold is not easy. The structures, especially in smaller species, are minute, requiring dissecting microscopy to ensure correct anatomical extraction. However, in both the rabbit and the human, the structures are visible without magnification. Although the structures may be easily identified, it is difficult to avoid cellular contamination from both the supraglottis and subglottis. This anatomic contamination may prove detrimental to this type of investigation. Although fibroblasts, by definition, are the heartiest and most plentiful cell in the body, fibroblast populations appear to respond differently

based on location. In fact, vocal fold fibroblasts have been shown to be very different from fibroblasts harvested from the subglottis with regard to extracellular matrix gene stability (Thibeault, Li et al., 2002). In addition, vocal fold fibroblasts seem much more challenging to grow and culture than skin fibroblasts. Our laboratory has extensive experience with fibroblasts from various regions and without exception, vocal fold fibroblasts are more challenging to cultivate. Although the current study sought to determine a confirmatory protocol for homogeneity of fibroblast culture, no standardized harvest technique has been described. The development of such a protocol would be invaluable to future investigation utilizing *in vitro* methods to describe vocal fold fibroblasts.

Within the vocal folds, fibroblasts reside in relatively close proximity to epithelial cells, endothelial cells, and skeletal muscle cells. Ideally, visual inspection of the culture could confirm fibroblasts versus other cell types. However, more objective means of cell identification are necessary. The current study developed a protocol based primarily on collagen synthesis. The literature is full of reports describing collagen synthesis in both fibroblasts and endothelial cells. Therefore, based on a myriad of methods, collagen synthesis can rule out the presence of skeletal muscle cells. Assuming collagen synthesis, positive staining for vimentin and negative staining for von Willebrand differentiated fibroblasts and endothelial cells. This protocol clearly defined the fibroblast population in the current study.

However, this protocol was limited to human lines based on the von Willebrand Factor antibody. The antibody was created in rabbit. Several attempts were made to stain RVFF with von Willebrand Factor at a variety of concentrations. Unfortunately, diffuse non-specific staining was noted in all cases. Furthermore, control endothelial cells for the rabbit model were difficult to obtain. Therefore, confirmation of fibroblasts in RVFF culture was less complete.

Although great certainty regarding fibroblasts in culture was obtained, objective data can not fully confirm this observation. Although unlikely, the discrepancy between RVFF and HVFF may be associated with differences in cell types used for experiments.

The discrepancy between HVFF and RVFF inflammatory gene expression is interesting. In addition, HVFF and RVFF appear to respond differently to mechanical stress. The preliminary experiments using RVFF showed massive suppression of iNOS expression in response to low levels of CTS. In response to 3% mechanical stress, RVFF iNOS expression was suppressed approximately 90%. Although iNOS was not targeted, this level of suppression was not achieved in HVFF. This discrepancy may further confirm interspecies variability. The vocal folds are highly specialized structures in all species. However, in humans, the vocal folds are critical to communication. Although the mode of rabbit communication has not been clearly identified, it appears clear that vocalization is not the key element. Species-specific differences in vocal fold function provide a potential explanation for the discrepancies encountered in the current study. In addition, previous investigations utilizing an *in vitro* model to describe vocal fold fibroblasts utilize a canine model. Although the structure of the canine vocal fold has been described as a variant of the human fold (Garrett, Coleman, & Reinisch, 2000), perhaps function is more relevant. Clearly, dogs utilize their vocal folds to a greater extent than rabbits for communication. Therefore, the canine response to inflammatory stimuli may be more similar to the human response.

Another potential reason for the discrepancies in the results may be the passage of cells used. Beyond passage six, human vocal fold fibroblasts have been shown to be relatively unstable in terms of extracellular matrix gene expression (Thibeault, Li et al., 2002). To date, no investigations have focused on other gene expression as a function of cell doubling. The RVFF

cells used in the current study were P3. In contrast, the HVFF were P6. Interestingly, several preliminary studies were attempted with later passage RVFF (P5-P6) cells. Upon Bioflex plating, these cells became senescent. RNA extraction of the cells revealed cell death. The passage of vocal fold fibroblasts appears to be a critical variable for investigation of this type. In addition, the later passage cells provided by Dr. Thibeault had a much lower proliferative rate than younger HVFF cultures. The relationship between cell doublings and cell activity in vocal fold fibroblasts warrants further investigation.

Regardless of the potential interspecies variability, inflammatory gene expression was suppressed following four hours of mechanical stimulation in the human cell line. Although the pathway associated with this finding was not elucidated in the current project, it has been described in other tissues. As mentioned previously, low levels of mechanical stress appear to limit IκB degradation in response to inflammatory stimuli. When IκB is degraded, NF-κB is permitted to translocate into the nucleus and stimulate pro-inflammatory gene expression (Deschner et al., 2003) (Figure 17). This pathway is a target of future investigation. Furthermore, high-magnitude cyclic stress is proinflammatory, and when applied in combination with IL-1β, the effects are additive and stimulate an inflammatory response (Agarwal et al., 2003). Although magnitude typically refers to the degree of equibiaxial stress applied to the monolayer, it is hypothesized that a temporal variable is likely also involved. This temporal hypothesis would provide a rationale explanation for the current data.

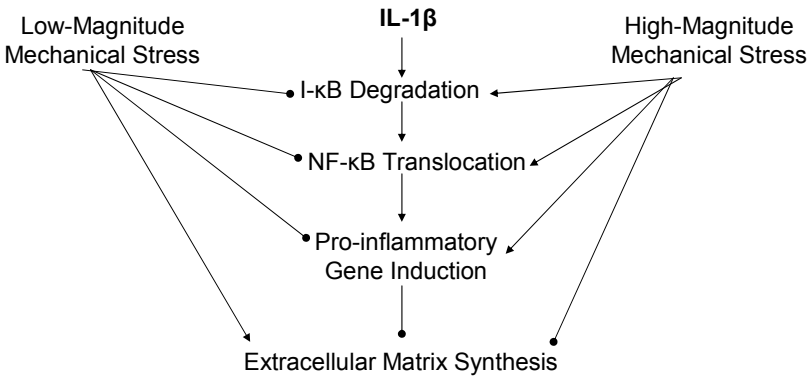

Figure 17. Magnitude of CTS and inflammation

Arrows indicate excitatory pathways; rounded lines are inhibitory. Adapted from Deschner, Hoffman, Piesco, & Agarwal (2003).

Twenty-four hours of mechanical stress appears to surpass the threshold to elicit an injurious response. At the cellular level, the response to prolonged mechanical stress was variable. However, consistently increased levels of the pro-inflammatory cytokine inteleukin-6 and the lipid inflammatory mediator PGE-2 were noted following 24 hour CTS exposure. In addition, the *threshold* hypothesis is confirmed by inflammatory gene expression in response to mechanical stress alone at 24 hours. Both COX-2 and MMP-1 gene expression substantially increased following 24 hours of stress alone (without IL-1β stimulation). This phenomenon was not noted in the 4-hour experiments. Clearly, sometime between 4 and 24 hours of CTS exposure, the phenotype of human vocal fold fibroblasts became catabolic. Characterization of this threshold should be a target of future investigation. Ideally, therapeutic management of patients with voice disorders should, at the very least, consider these issues in the development

of treatment programs. Furthermore, this finding coincides well with clinical and common sense. Voice use for an extended amount of time is likely to yield dysphonia.

Furthermore, the relationship between the in vitro environment proposed in the current study and real tissue dynamics are tenuous at best. The Flexcell unit, although tremendously popular, clearly does not simulate phonation. The relative frequency of mechanical stress created by the unit is not even close to the human condition. However, the cellular response to phonation has not been elucidated. Titze and colleagues (2004) recently created a bioreactor that is hypothesized to stimulate oscillatory stresses. They recently subjected a single human cell line to these stresses and found that ECM gene expression was altered in the context of prolonged mechanical stress. These data likely confirm that the present study did not surpass the threshold to elicit a change in ECM synthesis.

An overwhelming limitation of the current study is the use of an *in vitro* system. Clearly, these data should be considered a platform on which to base future investigation, not an absolute finding. The *in vitro* environment is falsely controlled yielding limited applicability to the human condition. However, the use of *in vitro* methods improves insight into the mechanics of human behavior, thereby contributing substantially to the body of work regarding laryngeal mechanics.

More broadly, the goal of the current series of experiments was to determine whether or not vocal fold fibroblasts respond similarly to other cells throughout the body with regard to the effects of mechanical stress in the context of inflammation. The current results may suggest a role of some sort of phonation in the context of acute inflammation of the vocal folds. If homologous to other tissue, the simple act of phonating could limit the inflammatory response. Furthermore, therapeutic tasks that modulate the impact stresses between the vocal folds during

phonation could be utilized to specifically alter the wound healing cascade and promote more biomechanically sound tissue in the long term. These issues are currently being addressed in a human model of phonotrauma. However, no investigation has examined the response at the cellular level. The results of the current study suggest that the vocal folds may benefit from mechanical stress under some conditions. However, the *threshold* concept that was introduced in previous sections must be accounted for in clinical voice care.

The concept of vocalization reducing inflammation is paradoxical to the traditional clinical concept of managing patients with acute vocal fold injury. Traditionally, these patients were treated via the vocal diet approach, limiting voice use (Roy et al., 2001). This treatment approach, which remains common to this day, disregards the vast body of literature suggesting potentially detrimental effects of immobilization following acute injury. Perhaps the most well-researched area of medicine with regard to this phenomenon is orthopedics. The orthopedic literature eloquently describes the potentially deleterious effects of immobilization which include tissue degradation and altered function. This literature was reviewed at length in the Introduction. Although comparing the meniscus of the knee joint to the vocal folds is tenuous, the data are overwhelming suggesting a critical role in some degree of mobilization following injury.

Mobilization following injury has been shown to not only limit the inflammatory response, but also improve the structure of the injured tissue in the long term. Given that the inflammatory response orchestrates the subsequent wound healing events including the formation of new extracellular matrix formation, it makes sense that attenuation of the inflammatory response would thereby alter the composition of the tissue. The current study sought to elucidate this trend in the vocal folds. To that end, gene expression data were analyzed

with regard to known extracellular matrix components in the vocal folds. Particular emphasis was dedicated to glycosaminoglycans and proteoglycans (HA and decorin) which, in combination with collagen, appear to be mainly responsible for the biomechanical properties of the vocal folds. Unfortunately, the present data failed to find evidence that mechanical signaling alters ECM synthesis.

This finding is likely related to the relative timing of data collection. Although Xu and colleagues (2000) reported significantly decreased proteoglycan synthesis as early as 24 hours after sustained exposure to IL-1β, perhaps increased duration of exposure is required for the same finding in vocal fold fibroblasts. Similiarly, a possible hypothesis is that vocal fold fibroblasts do not alter ECM synthesis in response to relatively short-term (24 hours) mechanical stress. Experiments in the literature investigating proteoglycan concentration *in vivo* all used much later timepoints to harvest tissue. Therefore, increased duration of mechanical stress may be required to stimulate alterations in ECM. This hypothesis is problematic, however. The current data suggest that 24-hours of mechanical stress did not attenuate the inflammatory response. In contrast, it appeared to worsen inflammation. Therefore, the duration of exercise that may increase proteoglycan synthesis may also facilitate a catabolic inflammatory response in the vocal folds. However, strenuous levels of exercise have been shown to decrease proteoglycan synthesis. Unfortunately, the current studies did not appear to capture the dynamics of ECM synthesis. It is interesting, however, that the initiation of a catabolic response with regard to the inflammatory phenotype did not appear to affect ECM synthesis.

An alternative hypothesis is that VFF may be resistant to inflammation-induced EMC alterations. For example, models of arthritis have shown that prolonged exposure to inflammatory stimuli leads to the tissue degradation associated with the clinically relevant

complaints associated with the disease process (Agarwal et al., 2001). In addition, IL-1β has been shown to decrease proteoglycan synthesis as early as 24 hours in chondrocytes (Xu et al., 2000). These data may suggest that the vocal folds have an increased tolerance of inflammatory stimuli with regard to extracellular matrix synthesis. Furthermore, it may be hypothesized that a separate threshold for ECM may be present in the vocal folds. For example, an inflammatory stimulus such as a brief episode of phonotrauma may surpass the threshold for a wound healing response, resulting in inflammation. However, this single event is unlikely to elicit a morphological alteration in the extracellular matrix. Clinically, this explanation seems rational. Patients present with acute voice problems associated with vocal fold inflammation. Clearly, these patients have surpassed the threshold to stimulate a relevant inflammatory response to ensure immune competence and facilitate tissue repair. However, this single event is unlikely to cause permanent structural alteration to the lamina propria.

Therefore, although the lack of findings regarding the impact of CTS on ECM synthesis may seem disappointing, these data may shed increased insight into the possible inherent resiliency of the vocal folds. Because the vocal folds must undergo marked mechanical stress associated with phonation and other vocal, vegetative tasks, degradation of the LP associated with single inflammatory events could potentially impact vocal function over the course of the lifetime.

Although much information can be gleaned from the current investigation, a source of criticism regards the use of a single line. The difficulties associated with attaining normal, healthy human vocal fold fibroblasts were described in the Methods section. Inclusion of even one more cell line would be extremely helpful in validating the current findings. Furthermore, given the gender differences associated vocal fold morphology described by Gray (1997), future

investigations should examine potential gender-derived cellular differences. Because increased amounts of HA have been identified in the LP of males, perhaps these cells are phenotypically different and would therefore respond differently to both inflammatory stimuli and mechanical stress.

Overall, the present results suggest that there appears to be a positive role for some level of mechanical signaling in vocal fold inflammation. The current study, with its inherent limitations, provides a foundation for future work in this area. The laryngology community appears to be several decades behind the orthopedic community with regard to the basic science behind rehabilitation. This lag may be due to the anatomical limitations of the organ of interest. Clinicians lack the ability to "lay hands" on the larynx and vocal folds, unlike physical therapists or orthopedic rehabilitation specialists. However, it is hoped that more basic biological research will infuse into the field of communication sciences. Education regarding biological processes must become common in graduate curriculum, adding to education in behavioral science. The broad impetus of the current project was to cross the bridge between basic and behavioral science.

APPENDIX (RAW ELISA DATA)

21. BCA Assay Data

Condition	Mean (mg)	Standard Error of the Mean
Control	5.01	0.14
Interluekin-1β	5.60	0.42
Stress	6.03	0.28
Stress + IL-1β	5.80	0.14

22. Prostaglandin-E2 ELISA Data

Condition	Mean (pg/mg protein)	Standard Error of the Mean
Control	92.55	62.21
Interluekin-1β	364.07	223.14
Stress	34.29	4.78
Stress + IL-1β	1195.00	4.77

23. Interleukin-6 ELISA Data

Condition	Mean (pg/mg protein)	Standard Error of the Mean
Control	0.12	0.02
Interluekin-1β	128.87	37.06
Stress	0.19	0.07
Stress + IL-1β	940.05	410.63

24. Collagen ELISA Data

Condition	Mean (pg/mg protein)	Standard Error of the Mean
Control	746.83	35.94
Interluekin-1β	798.17	62.18
Stress	749.81	42.94
Stress + IL-1β	786.44	79.87

25. Hyaluronic Acid ELISA Data

Condition	Mean (pg/mg protein)	Standard Error of the Mean
Control	216.10	18.29
Interluekin-1β	236.28	16.58
Stress	150.26	10.92
Stress + IL-1β	208.06	44.93

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