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Characterization of vocal fold scar formation, prophylaxis and treatment using animal models

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Abstract

Purpose of review—This article reviews recent literature on animal models used to study the pathogenesis, detection, prevention and treatment of vocal fold scarring. Animal work is critical to studying vocal fold scarring because it is the only way to conduct systematic research on the biomechanical properties of the layered structure of the vocal fold lamina propria, and therefore develop reliable prevention and treatment strategies for this complex clinical problem.

Recent findings—During the period of review, critical anatomic, physiologic and wound healing characteristics, which may serve as the bases for selection of a certain species to help answer a specific question, have been described in mouse, rat, rabbit, ferret and canine models. A number of different strategies for prophylaxis and chronic scar treatment in animals show promise for clinical application. The pathways of scar formation and methods for quantifying treatment-induced change have become better defined.

Summary—Recent animal vocal fold scarring studies have enriched and confirmed earlier work indicating that restoring pliability to the scarred vocal fold mucosa is challenging but achievable. Differences between animal models and differences in outcome measurements across studies necessitate considering each study individually to obtain guidance for future research. With increased standardization of measurement techniques it may be possible to make more inter-study comparisons.

Keywords

animal models; larynx; prophylaxis; tissue regeneration; vocal fold; wound healing

Introduction

Vocal fold vibration is the source of voice production, which is directly driven by the biomechanical properties of the layered vocal fold lamina propria. Scarring disrupts this layered structure and causes significant change in vocal fold tissue biomechanics, resulting in a range of voice problems that often significantly compromise patient quality of life. The degree of scar-induced dysphonia is thought to relate to the location and magnitude of the scar. A myriad of treatments have been explored but no single treatment has emerged as a reliable remedy. This may be due, at least in part, to difficulty studying scar prevention and treatment in humans; a consequence of individual variability in scar formation and treatment response. Animal models present an opportunity to systematically study prevention and treatment, however, no single animal has emerged as the ideal model because of differences

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in vocal fold size, layer structure and extracellular matrix (ECM) components among animals, and because of differences in research questions and methodological approaches across studies.

Selection of the most appropriate animal model depends on the question being addressed and requires information about the characteristics of normal and scarred vocal folds in animals and humans. The characteristics of particular interest that are related to vocal fold scarring include size, shape, structure, presence/absence of a vocal ligament, organization and relative abundance of ECM components (e.g., hyaluronic acid [HA], collagen types I and III, fibronectin), native biomechanical properties, and the degree to which the animal normally vocalizes. Some of these critical characteristics have been described for mice, rats, ferrets, rabbits, dogs, sheep and pigs [1–18] and in many cases form the primary bases for selection of a specific species to help answer a specific research question. However, the choice of animal model is not always based on physical characteristics alone as there are often practical issues to consider. Such practical considerations include animal size, facilities available, and the cost of purchasing and maintaining the animals for the duration of the study. In some cases, practical considerations dictate that researchers compromise on selection of an ideal model.

Regardless of the question being addressed or the animal selected to aid in answering the question, animals have become increasingly important in research concerned with the prevention and treatment of vocal fold scarring. This paper reviews scarring animal studies conducted during 2009–2010. During this period, mouse, rat, rabbit, ferret and canine models were used to further understanding of the pathophysiology of scarring, to develop assessment and measurement tools, and to evaluate pre-clinical experimental treatments.

Characterization of Scar Pathophysiology

Central to the use of any animal model for research in vocal fold scarring is characterization of the structure of the vocal fold and phases of wound healing. Ling et al. [19] described the gross anatomy of the rat vocal fold mucosa and early changes in cellular morphology, density and distribution following unilateral vocal fold injury. Dramatic changes in lamina propria and epithelial cell composition, morphology, and density were observed during the first seven days post-injury. Re-epithelialization and normalization of mucosal area/volume occured within three days post-injury. Routine histologic analyses revealed the sequential recruitment of neutrophil-like cells, epithelial cells and fibroblast-like cells. The researchers suggested that these sequential cellular alterations may underpin functional alterations, and form the bases of undesirable tissue repair. They concurred with previous work indicating that vocal fold scar formation in rats occurs more quickly than in larger mammals, and that while the rat shares many anatomical, cellular and extracellular features with the human vocal fold, it is generally a simpler structure in a different biomechanical environment.

Another recent study characterizing wound healing in the injured rat vocal fold was conducted by Ohno et al. [20]. These authors focused on transcription of transforming growth factor β-1 (TGF-β1), hepatocyte growth factor (HGF), and the HGF receptor c-Met, during the inflammatory, proliferative, and remodeling phases of wound healing. Results revealed time-dependent changes: TGF-β1 expression increased significantly on day seven post-injury compared to control, HGF expression decreased significantly on day one and increased significantly on day 14 post-injury, and c-Met expression decreased significantly on days one, three and 56 post-injury, and increased significantly on day 28 post-injury.

Capitalizing on published research reports using a rat model, Li et al. [21] developed a computational agent-based model (ABM) model to simulate inflammation and healing in surgically injured vocal folds. In these simulation studies, HA deposition was enhanced by

inflammation and, in turn, induced ongoing inflammation. The authors interpreted this finding to suggest that HA fragments might be the clinical surrogate of tissue damage. The comprehensive summary tables provided in this article offer a useful resource for future researchers using animal models to study treatment effects in the acute stage of wound healing.

Gene knockout, knockdown, and knock-in models have not been exploited in vocal fold scar research to date; however, their potential value makes the mouse an attractive animal model. A central challenge in the utilization of mice in vocal fold scar research is the reproducible surgical manipulation of such small laryngeal structures. As a precursor to experimentation with genetically engineered mice, Yamashita et al. [17] developed a surgical procedure to create vocal fold stripping injuries in wildtype mice. Custom fabricated laryngoscopes and micro-surgical instruments were used. The injured vocal fold mucosa was largely absent one day post-injury but completely reepithelialized by seven days post-injury. Follow-up work using this model demonstrated ECM changes generally comparable to those reported in larger animal models and humans [18], suggesting that despite its small size, the mouse model is a relevant tool for vocal fold scarring research.

Assessment/Evaluation of Scar Formation

Varying methodologies (e.g., different animal models, different time periods, different analysis techniques) often make comparisons between studies difficult. Jabbour et al. [22] attempted to help reduce this problem by developing a standard method for quantifying the geometric properties of the medial vibratory portion of the canine vocal fold and further suggested that future research replicate this method in other animal models. Such standard procedures are necessary to enhance our ability to make reasonable comparisons and therefore aid our understanding of the pathophysiology of scar formation. Many of the research methodologies routinely employed in clinical studies, such as utilizing standardized measures, reporting reliability, and blinding judges making perceptual decisions, are often omitted in animal studies.

One factor inhibiting understanding of the pathophysiology, prevention and treatment of scar formation is the limitation of currently available imaging procedures. Videostroboscopy, high speed imaging, kymography, ultrasound and other traditional radiological technologies do not permit visualization of vocal fold tissue architecture at the micron level. While histological assays provide powerful information they cannot be performed on living humans with intact larynges, and when used in animals necessitate sacrifice at different time points. Thus, histology does not permit the sequential measurement of tissue response in the same animal over time, which is highly desirable when studying treatment effects. These limitations motivated Herrera et al. [23] to conduct a proof of concept imaging study using ex vivo ferret and canine larynges. Using 11.7 Tesla magnetic resonance micro-imaging (MRI) with 39-μm/pixel resolution, they successfully identified epithelium, lamina propria, muscle, and cartilage in unscarred ferret and canine vocal folds, and the presence of scar tissue in ferret vocal folds. Equally important, they accurately detected HA-based implants injected into both ferret and canine vocal folds. Imaging results were confirmed with histology. The authors argued that this MRI imaging modality offers enhanced efficiency over histological preparation. Once developed, in vivo high-resolution MR serial imaging has the potential to offer insight into differential changes in pathogenic events such as scar formation, and reduce the number of research animals needed in future studies.

Welham et al. [12] developed and evaluated a rat excised larynx model for measures of vocal function that reflect biomechanical changes resulting from treatment. Rats were

assigned to one of four experimental groups: chronic vocal fold scar, chronic vocal fold scar treated with 100-ng basic fibroblast growth factor (bFGF), chronic vocal fold scar treated with saline (sham treatment), and unscarred untreated control. Phonation threshold pressure, glottal resistance, glottal efficiency, vibratory amplitude, and vibratory area were used as dependent variables. Phonation was achieved, and data were collected from all control and bFGF-treated larynges; however, phonation was not achieved with three of six chronically scarred and one of six saline-treated larynges, suggesting significantly increased mucosal stiffness in these cases. Vocal function indices were sufficiently sensitive to reflect deterioration in vocal function in the scar condition and treatment-induced change in the bFGF condition. Whether the improvements seen in the sham group represented an artifact or therapeutic benefit could not be determined. Nevertheless, this model appears promising as a tool to study the functional characterization of biomechanical tissue changes resulting from vocal fold scar, as well as the evaluation of experimental therapies.

Treatment

Treatment research has generally focused on either prophylactic approaches to the prevention or attenuation of scar formation post-injury, or restoration/regeneration of vibratory function in cases of chronic scar. In these regards, several advances have been made during the 2009–2010 period of review.

Prophylactic treatments

Prophylactic treatments are based on the notion that the acute healing phase represents a critical period during which therapeutic intervention may minimize eventual scar formation. Because migrating inflammatory cells and resident fibroblasts begin to synthesize ECM 2–3 days following injury [4, 5], these interventions are generally administered at the time of the injury. Mesenchymal stem cells (MSCs), Vitamin A, corticosteroids, HA-based biomaterials, decellularized scaffold materials, and HGF have been used in animal models during the past year.

MSCs have received significant attention in regenerative medicine due to their apparent immunoprivilege and differentiation capacity. Svensson et al. [24] recently evaluated healing outcomes following human MSC delivery at the time of rabbit vocal fold injury. Animals were placed on immunosupression for the duration of the experiment. Histologic and rheologic assays were performed three months post-injury and revealed reduced collagen abundance, reduced lamina propria thickness, and improved dynamic viscosity compared to saline-treated controls. These promising findings align with earlier work published by this research group and others [25, 26].

Vitamin A holds demonstrated importance in the development and maintenance of the vocal fold epithelium [27, 28]. In an attempt to evaluate the potential therapeutic value of this agent during vocal fold wound healing, Akdogan et al. [29] applied 0.025% topical retinoic acid gel to rabbit vocal folds at the time of injury. Histological analyses, performed 10 days post-injury for both experimental and control animals, revealed less collagen accumulation in the treatment group compared to saline-treated controls. Based on these results, the authors suggested that topical vitamin A application could be easily used in humans at the time of surgery to facilitate enhanced wound healing.

Corticosterioids are believed to influence wound healing by modulating the synthesis and maturation of collagen, inhibiting fibroblast proliferation, and suppressing the antibacterial and phagocytic action of certain immune cells. Consequently, they are frequently employed in conjunction with phonosurgery, with varying degrees of success. To better understand the mechanism and potential value of corticosteroid delivery at the time of vocal fold surgical

injury, Campagnolo et al. [30] evaluated histological outcomes three and seven days post-injury in a rabbit model. Cell infiltration was similar in corticosteroid-treated and control vocal folds; however, collagen deposition was significantly reduced in the corticosteroid-treated group. These results align with evidence that inflammatory cells and fibroblasts begin synthesizing ECM 2–3 days post-injury [4, 5] and that HA, collagen, and fibronectin deposition is most prominent at 3–5 days post-injury [13]. The authors concluded that their data support a positive therapeutic effect following corticosteroid delivery during the acute phase of wound healing; additional work evaluating whether this benefit persists into the later phase of ECM remodeling (leading to eventual scar attenuation) would provide additional value.

HA plays an important role in wound repair, hydrodynamics, cell proliferation and cell migration and has been the focus of a number of vocal fold scarring studies. Recently, Thibeault et al. [31] used a rabbit model to determine if injection of a chemically modified HA-based scaffold at the time of vocal fold injury would facilitate improved wound repair and preservation of near-native viscoelasticity. Animals were sacrificed and vocal fold tissues were evaluated six months post-injury. Scaffold-treated vocal folds exhibited downregulation of fibronectin, fibromodulin, procollagen I, HA synthase and TGF-β1 transcription compared to saline-treated controls. Rheologic analysis revealed improved viscous and elastic shear moduli in treated vocal folds compared to controls. These findings indicate that the early therapeutic benefits of prophylactic HA delivery, previously reported up to 21 days post-injury [32], are maintained through the remodeling phase of chronic scar formation.

Several recent reports have evaluated the use of decellularized scaffold materials to assist acute vocal fold repair. Gilbert et al. [33] implanted decellularized porcine liver ECM (a tissue rich in endogenous HGF) following vocal fold injury creation, in a canine model. Three months post-injury, the scaffold-treated vocal folds exhibited greater collagen abundance (including a greater ratio of collagen type III to type I), comparable elastin abundance, and comparable glycosaminoglycan (GAG) abundance, compared to untreated injury controls. Elastin abundance was greater and GAG abundance was less than in experimentally naïve control tissue. The authors acknowledged that their ECM scaffold did not appear to promote vocal fold regeneration, but argued that the general therapeutic strategy is worthy of ongoing investigation.

In a similar study, Xu et al. [34] implanted decellularized bovine vocal fold mucosa following cartilaginous vocal fold injury in a rat model. Histologic analyses revealed an initial increase in collagen type I, type III and GAG abundance compared to untreated controls (three days post-injury), followed by a gradual decrease over the following three months. The scaffold was eventually degraded by the host with no evidence of chronic fibrosis or calcification. In a follow-up study utilizing the same animal model, the authors evaluated the potential of the decellularized bovine scaffold as a timed-release system for HGF delivery in vivo [35]. Initial in vitro experiments confirmed sustained HGF release by HGF-loaded scaffolds over seven days. Implanted HGF-loaded scaffolds demonstrated fewer inflammatory cells three days post-injury, and less type III collagen and HA abundance at seven days post-injury, compared to non-HGF scaffold controls. Complete scaffold degradation was again observed by three months. Although these studies utilized cartilaginous rather than membranous vocal fold scaffold implantation, it appears that this decellularized bovine scaffold holds therapeutic promise as a strategy for the exogenous delivery of select growth factors in vivo.

Treatment of actively remodeling and chronic scar

If sufficient advances are made with prophylactic measures, the need to treat chronic scars may, in the future, become rare. However, at the present time, chronic scarring remains a challenging clinical problem. Thibeault et al. [36] used a rabbit model to evaluate the performance of three different biomimetic approaches for scarred vocal fold regeneration. Two months post-injury (mature scar formation requires approximately six months in the rabbit model [37]), vocal folds were treated with either autologous fibroblasts, an HA-based synthetic scaffold material, or autologous fibroblasts encapsulated in the scaffold. Histologic and rheologic assays were performed two months following treatment. Injured vocal folds treated with autologous fibroblasts demonstrated a superior outcome, suggesting that improved understanding of cell-scaffold interactions is needed to optimize the use of scaffold-based strategies in vocal fold regeneration. In another recent study performed by this group using a similar experimental approach, mouse bone marrow derived-MSCs (BM-MSCs) encapsulated in a synthetic scaffold demonstrated a superior treatment outcome compared to BM-MSCs alone, when injected into rat vocal folds one month post-injury (mature scar formation requires approximately two months in the rat model [14]). Gene transcription and immunohistochemical assays were performed one month following treatment. The difference in outcomes between these studies might relate to the different cell type injected, different animal model, different treatment and measurement time points, and/or different experimental assays employed.

bFGF may accelerate wound healing via stimulation of fibroblast proliferation and angiogenesis. In an extension of previous research focused on growth factor therapy for vocal fold scarring, Suehiro et al. [38] treated actively remodeling vocal fold scars with repeat bFGF injections, using a canine model. bFGF injections were performed four and five weeks post-injury (mature scar formation requires approximately six months in the canine model [39]); excised larynx and histological analyses were conducted six months post-injury. bFGF-treated vocal folds exhibited less tissue contraction, greater GAG abundance, lower phonation threshold pressures, and greater vibratory amplitude, compared to the saline-treated group. The authors appeared to view this as a pre-clinical study, noting that (unlike HGF) bFGF holds a relatively safe profile across a range of doses, and is currently approved for clinical use in Japan. Of note, this research group also recently reported the first successful human case report using bFGF therapy for age-related vocal fold atrophy [40].

Kishimoto et al. [41] also utilized a canine model in an attempt to treat chronic vocal fold scar using hydrogel-based HGF delivery. Vocal folds were six months post-injury at the time of treatment; histologic and excised larynx outcomes were measured three months following treatment. The HGF-treated group demonstrated improved mucosal wave excursion, reduced phonation threshold pressure, reduced collagen abundance, and improved HA abundance, compared to hydrogel-saline (i.e., no HGF) control. The authors concluded that HGF holds potential for the treatment of chronic vocal fold scar, particularly when combined with a therapeutic scaffold material as reported in this study.

Conclusions

Animal models represent an essential and valuable tool when studying the pathogenesis, detection, prevention and treatment of vocal fold scarring. Although there is no ideal animal for addressing all possible scientific questions, animal models provide a means to systematically address scarring issues in ways that cannot be done in human patients. Studies reported during the 2009–2010 period of review used mouse, rat, rabbit, ferret and canine models. Recent work has provided improved knowledge of the wound healing processes that culminate in vocal fold scar formation, the potential benefit of a number of

cell, scaffold and growth factor therapies, and emerging methodologies for quantifying treatment-induced change.

Despite the value of animal models, recent research in this area is not without limitation. Beyond the obvious issue of interspecies variation, there is often little consideration of age- and sex-related influences on wound healing. These factors have been associated with ECM differences in the human lamina propria [42, 43], and therefore may also be important modulators of healing outcome. Further, the increased application of standardized assays across studies would be beneficial in allowing comparisons and reducing the need for redundant experiments. Assays dependent on visual or auditory-perceptual judgments should consistently mask judges and include repeat measures to allow the reporting of reliability data. Whereas almost all animal studies evaluated in the period of review used histologic approaches to evaluate general morphology and protein distribution within the ECM, fewer studies utilized excised larynx or rheologic approaches to examine vocal fold vibratory function or viscoelasticity. No studies reported in vivo phonation. Ideally, studies should evaluate both biologic (i.e., cell, transcript, protein, tissue) and functional (i.e., viscosity, elasticity, phonatory) features of interest, especially when evaluating pre-clinical experimental therapies. Attention to these issues should further improve the applicability of animal studies to the characterization, prevention and treatment of vocal fold scar.

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