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Glucocorticoids regulate extracellular matrix metabolism in human vocal fold fibroblasts

Hang Zhou, M.D.¹, Mahalakshmi Sivasankar, Ph.D.², Dennis H. Kraus, M.D.³, Vlad C. Sandulache, M.D., Ph.D.⁴, Milan Amin, M.D.¹, and Ryan C. Branski, Ph.D.¹

¹ Department of Otolaryngology New York University New York, NY

² Speech, Language, and Hearing Sciences Purdue University West Lafayette, IN

³ Head and Neck Surgery Memorial Sloan-Kettering Cancer Center New York, NY

⁴ Bobby R. Alford Department of Otolaryngology-Head and Neck Surgery Baylor College of Medicine Houston, Texas

Abstract

Objectives/Hypothesis—Given the recent emergence of encouraging efficacy data regarding the utility of intralesional glucocorticoid (GC) injection for a variety of vocal fold pathologies, we sought to describe the location and expression pattern of the GC receptors within the vocal folds and quantify the effects of GCs on vocal fold fibroblasts.

Study Design—In vitro, in vivo

Methods—Immunolocalization of the GC receptor (GCR) was performed on normal rat vocal fold tissue. Receptor expression was also assayed in our human vocal fold fibroblast cell line. These cells were then treated with exogenous dexamethasone (DM) to quantify the effects of GCs on receptor expression, proliferation, TGF- β -induced collagen secretion, and matrix protease synthesis.

Results—Positive immunostaining for the GC receptor was found throughout the vocal fold with particularly strong staining in the epithelium and capillaries. Human vocal fold fibroblasts constitutively express the GC receptor, but this expression decreased in response to exogenous DM. DM also decreased fibroblast proliferation and TGF- β -induced collagen synthesis. DM also abrogated TGF- β -mediated effects on enzymes related extracellular matrix turnover.

Conclusions—Our data are the first to provide mechanistic insight regarding the recently-published favorable data regarding the utility of GCs in patients with vocal fold scar. Although further investigation is warranted, both the accessibility of this class of agents and the amenability to office-based procedures are likely to direct patient care models.

Level of Evidence—N/A

Keywords

vocal fold; voice; steroids; scar; dexamethasone; glucocorticoids

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Address all correspondence to: Ryan C. Branski, Ph.D. Department of Otolaryngology, New York University School of Medicine 550 First Avenue, 3C, New York, NY 10016 Phone: (212) 263-3705 Fax: (212) 263-8490 ryan.branski@nyumc.org.

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Conflict of Interest: None

INTRODUCTION

Though many have outlined perpetual disappointment regarding the lack of efficacious therapies for patients with vocal fold scar, emerging, yet limited clinical data may suggest otherwise. A recent retrospective cohort study reviewed outcomes in 34 patients with a variety of vocal fold pathology who underwent intralesional glucocorticoid (GC) injections. Upon further inspection of these data, 16 patients with vocal fold scar and/or nodules, both fibroplastic tissue phenotypes, were included (not all mucosal lesions). Of these 16 patients, 15 improved significantly. The sole failure had chronic laryngitis and keratosis requiring a biopsy.¹ These data concur with an earlier report describing a significant decrease in vocal fold nodule size in all patients following intralesional injection of steroids. Furthermore, the nodules completely resolved in 63 percent of patients in that series.² These preliminary efficacy data, combined with the recent trend towards office-based interventions in laryngology,³ provide substantial foundation for the systematic investigation regarding localized GC therapy, particularly in patients with vocal fold scar which has largely frustrated both clinicians and patients.

GCs are a member of the broad class of hormones and are utilized as both anti-inflammatory and immunomodulatory agents. Once GCs bind the glucocorticoid receptor, which belongs to the nuclear receptor family of ligand-dependent transcription factors, the activated complex acts upon a number of cells via both transcription and repression of numerous genes. GCs have been used extensively to treat hypertrophic scars and keloids with intralesional GC administration yielding a highly-variable response rate of 50 to 100 percent, and a recurrence rate of 9 to 50 percent.^{4–10} The mechanism is thought to be two-fold. First, GCs have been shown to be potent anti-inflammatory agents. Second, GCs have been recently shown to decrease collagen and glycosaminoglycan synthesis, in addition to decreasing fibroblast proliferation, and increasing hypoxia.^{6,11–16} GCs have been shown to interact with both transforming growth factor (TGF)- β and vascular endothelial growth factor, two mediators of fibroplasia.^{17,18}

In the larynx, these agents have been employed in the treatment of various inflammatory diseases including croup, laryngeal edema, and sarcoidosis.^{19–22} In addition, GCs have been shown to be effective in the management of patients with mild Reinke's edema.²³ A recent study attempted to determine the effects of post-operative healing in response to peri-operative steroid injection. Interestingly, no quantitative differences were observed with regard to the inflammatory response following injury. GC injection did, however, result in a significant decrease in the rate of collagen deposition out to 7 days following injury.²⁴ We, therefore, hypothesize that these outcomes are likely to be quite beneficial in patients with established fibrosis of the vocal folds and that the effects of GCs are likely related to regulation of extracellular matrix metabolism and fibroblast activation. As such, in the current study, we seek to provide mechanistic information regarding the GC receptors in the vocal folds and the effects of GCs on vocal fold fibroblast activity.

MATERIALS AND METHODS

Cell Model and Reagents

The HVOX human vocal fold fibroblast cell line was used in the current series of experiments at below passage 20.²⁵ Dexamethasone (DM) and TGF- β were purchased from Sigma Aldrich (St. Louis, MO) and R&D Systems (Minneapolis, MN), respectively. Concentrations of both DM and TGF- β ranged from physiological to likely supra-physiological.

Immunohistochemistry

Three, normal adult Sprague-Daley rats were euthanized (no treatment, no injury) and subjected to laryngectomy in order to immunolocalize baseline GC receptor status within the vocal fold mucosa. The tissue was processed and embedded in paraffin per standard protocols. Seven micrometer sections containing the bilateral vocal folds were then subjected to immunohistochemistry for the glucocorticoid receptor (rabbit polyclonal IgG, Santa Cruz Biotechnology, Santa Cruz, CA/1:100) with hemotoxin counterstain. Negative controls including the secondary antibody alone were performed (data not shown).

Western Blot

HVOX cells treated for 24 hours with DM at 10, 100, and 200ng/mL were lysed with M-PER (Mammalian Protein Extraction Solution-Pierce, Rockford, Ill). Equivalent amounts of proteins were separated by 7.5% SDS-PAGE gel and transferred to nitrocellulose membranes (Whatman, United Kingdom). The membranes were blocked overnight in 5% non-fat milk, and after rinsing, incubated at room temperature for one hour with the primary antibody (Santa Cruz Biotechnology, Santa Cruz, CA 1:1000). After washing, blots were subsequently incubated for one hour at room temperature with horseradish peroxidase-labelled secondary antibody (Bio-Rad Western Blot Kit, Bio-Rad Laboratories, Hercules, CA). The Bio-Rad Western Blot Kit was then utilized according to the manufacturer's recommended protocol to detect specifically labeled bands.

Collagen Secretion

Total collagen secreted into the culture medium was quantified using the Sircol Soluble Collagen Assay (Biocolor, Ltd, Ireland) as previously described by our laboratory.²⁶ Near-confluent HVOX, grown in 6-well plates, were serum-starved overnight and then treated with media containing 2% serum supplementation and TGF- β (10ng/mL) and/or DM at 50 and 200ng/mL for 24 hours and the post-culture medium was collected. The manufacturer's recommended protocol was then followed.

Fibroblast Proliferation

The CellTiter 96® Assay (Promega, Madison, WI), a modified MTT assay of cell metabolic activity and viability/cell number was employed to determine the effects of DM on HVOX cells. Subconfluent HVOX cells cultured in 96-well plates were serum-starved for six hours and then treated with media containing 2% serum supplementation and DM at 50, 100, and 200ng/mL for 48 hours. According to the manufacturer's protocol, 20 μ L of the Optimized Dye Solution was added to each well and incubated for four hours. Absorbance of solubilized formazan was then read at 570nm.

MMP-1/TIMP-1 Secretion

Secreted protease concentration was determined via commercially-available immunoassays (R & D Systems, MN). HVOX were grown to near confluence in 6-well plates and serum-starved for 24 hours. The cells were then treated with media containing 2% serum supplementation in addition to TGF- β (10ng/mL) and/or DM (50 and 200ng/mL) for 24 hours and the post-culture medium was collected. The manufacturer's recommended protocol was then followed.

Statistical Analyses

All experiments were performed in triplicate and the data are presented descriptively as the mean $\pm$ standard deviation. One-Way Analysis of Variance (ANOVA) was performed for each variable of interest using SPSS 12.0. Tukey HSD post-hoc analyses were performed if a main effect was significant at $p < 0.05$.

RESULTS

The GC receptor is constitutively expressed throughout the vocal fold

As shown in Figure 1A, diffuse staining was observed consistently throughout the rat vocal fold. Dense staining was observed throughout the vocal fold epithelium. Staining was less consistent in the lamina propria. However, positive mesenchymal cell staining was observed as well as focal, punctuate staining of the capillaries (Figure 1B).

Human vocal fold fibroblast expression of the receptor decreased in response to DM

Western blot analyses confirmed that human vocal fold fibroblasts constitutively expressed the GC receptor. As shown in Figure 1C (representative gel), basal GC receptor expression decreased in response to 24 hours of DM exposure in a dose-dependent manner.

DM limited TGF-induced collagen secretion and basal proliferative rate in vocal fold fibroblasts

Consistent with previous findings, TGF- β increased collagen secretion in our cells by approximately 80 percent (Figure 2A; $p < 0.05$). DM at low concentrations decreased this effect significantly and at increasing concentrations completely abrogated TGF- β -induced collagen secretion. In isolation, DM decreased basal levels of collagen secretion to below baseline. Similarly, DM decreased the basal proliferative rate of vocal fold fibroblasts, even at lower concentrations (Figure 2B).

DM and TGF- β affected MMP-1, but not TIMP-1 secretion in vocal fold fibroblasts

Commercial immunoassays were then employed to further investigate vocal fold fibroblast extracellular matrix metabolism in response to DM. As shown in Figure 3A, both TGF- β and DM in isolation, decreased baseline MMP-1 secretion by our cells. Interestingly, DM at increasing concentrations decreased the TGF- β -induced reduction in MMP-1 secretion. With regard to the inhibitor of MMP-1, TIMP-1, neither TGF- β nor DM had an effect (Figure 3B).

DISCUSSION

Scar is characterized by altered extracellular matrix (ECM) deposition and is often accompanied by a chronic inflammatory component. Therefore, therapies directed at modulating both the inflammatory response as well as ECM metabolism have a high likelihood of proving efficacious. Our data, in combination with recent animal work, suggest that glucocorticoids may have an effect on the ECM in the vocal fold.²⁴ Our data confirm that, in vitro, GCs may be anti-fibrotic as they limit vocal fold fibroblast activation (i.e., collagen secretion and proliferation). These effects are likely mediated via the GC receptor, which was localized to the epithelium and lamina propria of the vocal fold. This receptor determines the inherent sensitivity of a cell to GC. Cellular regulation of the GC receptor is related to adaptation to the hormone environment as well as varying biological requirements. In other systems, GC receptor levels have been shown to vary significantly due to various factors including age.²⁷ The current study only investigated a small number of animals with little age variability, a significant limitation that likely decreases the putative translation of the current findings to the clinic.

These receptors are thought to be regulated by both their own ligand (homologous regulation) as well as by other molecules (heterologous regulation). Consistent with our data in human vocal fold fibroblasts, GCs have been shown to be involved in the homologous down-regulation of the GC receptors.²⁸ The mechanism of this downregulation is unknown, but it is likely a component of a complex feedback network. In this regard, some

discordance in our data is also worth mentioning. As shown in Figure 1, positive staining for the GC α was only mildly impressive throughout the lamina propria of the vocal folds. However, our fibroblast cell line expressed the receptor protein at relatively high concentrations, potentially suggestive of some intra-species variability or other experimental confounds. These phenomena warrant further investigation as they may play a significant predictive role in determining the likely of favorable patient outcomes.

Immunolocalization of the GC receptor with high concentration within the epithelial layer may suggest a tertiary, yet favorable, outcome of localized GC therapy. Our laboratory has previously shown that a relatively inert hypertonic challenge to the vocal fold epithelium results in significantly compromised epithelial barrier function.²⁹ Likewise, chronic phonotrauma and/or surgical injury may also significantly compromise barrier function, placing the underlying lamina propria at risk.³⁰ Interestingly, numerous studies have shown that glucocorticoids improve barrier function via enhanced tight junction protein expression.³¹ Improved protection of the lamina propria may also contribute to enhanced healing, potentially shielding the underlying mucosa from the myriad of airborne irritants passing through the upper respiratory tract. Furthermore, we observed significant localization of the GC receptor to the capillaries within the vocal fold, consistent with other tissues. GCs have been shown to be a potent vasoconstrictor.³² Perhaps, a mechanism for the favorable outcomes shown clinically is this vasoconstriction, which has been previously hypothesized as a mechanism of angiolytic lasers in the context of non-hemorrhagic vocal fold lesions. We hypothesize that all of these factors likely are contributory to favorable clinical outcomes.

DM not only diminished TGF- β -mediated collagen synthesis and fibroblast proliferation, it had a regulatory effect on MMP-1, an enzyme responsible for extracellular matrix turnover. Cumulatively, these data may provide some mechanistic insight into the aforementioned animal study and the recently-published clinical efficacy data regarding localized injection of steroids for patients with frank vocal fold scar and vocal fold nodules. Matrix metalloproteinases (MMPs) are a family of enzymes classified according to their specificity for degrading different components of the extracellular matrix. TGF- β has been shown to decrease MMP-1 expression in various tissues, as shown in the current study of vocal fold fibroblasts. The role of MMPs in vocal fold injury and repair is relatively unknown and is based largely on conjecture from other tissues. However, our data suggest that not only are GCs regulating ECM synthesis, but also turnover in vocal fold fibroblasts.

In spite of the relatively rapid proliferation of innovative therapies of vocal fold scar, a frustrating clinical entity, satisfactory treatments are notably absent. Perhaps GCs which have been around for decades and may prove to be a useful and efficacious treatment modality for this otherwise recalcitrant patient population.

CONCLUSIONS

Our data are the first to provide mechanistic insight regarding the recently-published favorable data regarding the utility of GCs in patients with vocal fold scar, suggesting the GCs alter extracellular matrix metabolism in vitro. Although further investigation is warranted, both the accessibility of this class of agents and the amenability to office-based procedures are likely to direct patient care models.

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Figure 1.

Immunolocalization of the glucocorticoid receptor in the vocal fold (A; 20 $\times$). Dense, consistent staining was observed in the epithelium. Less positive staining was appreciated within the lamina propria. However, punctuate intracellular positivity is appreciated. Intense staining was also observed in the capillary space (B; arrows; 40 $\times$). Human vocal fold fibroblasts constitutively express the GC receptor (C; representative gel) and this expression consistently decreased with increasing concentrations of exogenous DM.

Figure 2. DM decreased TGF-β-induced collagen secretion human vocal fold fibroblasts in a dose-dependent fashion (A; * <0.05 relative to control condition, $^{\phi} <0.05$ relative to TGF-β treatment alone). DM also decreased basal proliferative rate in these cells (B; * <0.05 relative to control, $^{\circ} <0.05$ relative to DM 50).

Figure 3. DM altered the inherent fibroblast response to TGF- β with regard to MMP-1 (A), but not TIMP-1(B) secretion (* <0.05).