INTRODUCTION

Vocal fold (VF) scarring continues to pose a significant clinical challenge and is often associated with repeated phonotraumatic or inflammatory events. Scar and the associated tissue stiffness result in decreased vibratory pliability of the vocal fold mucosa and often underlie aberrant voice quality and resultant limitations in communicative effectiveness.1–3 Optimal treatment and strategies for prevention of VF scarring have not yet been established; however, direct glucocorticoid (GC) injection to focal regions of VF fibrosis has evolved as an increasingly common therapeutic option. Observational data suggest that these injections hold some degree of therapeutic potential,4 and intraoperative GCs are commonly employed under the assumption that they prevent scar formation following laryngeal surgery.5 However, the mechanism(s) underlying the antifibrotic effects of GCs remain unclear, and GCs are a large class of compounds with diverse pharmacokinetic properties. Clinically, no clear guidelines exist regarding GC use for VF scarring.6,7 The acquisition of preclinical, mechanistic insight into factors relevant to optimal therapeutic outcomes is critical to ultimately develop novel, targeted treatments for this challenging patient population.

In that regard, fibroblasts play a critical role in regulating extracellular matrix (ECM) turnover under normal conditions, and following injury, fibroblasts differentiate into myofibroblasts. This altered phenotype is associated with both increased metabolic and contractile properties. Excessive myofibroblast activity has been shown to mediate the development of fibrosis across tissue types, including the vocal folds.8 The stimuli for this differentiation is thought to be transforming growth factor (TGF)-β. The actions of TGF-β1 are mediated via a unique signaling pathway. Briefly, receptor-activated SMAD2 and SMAD3 are phosphorylated via TGF-β1 binding to the receptor and heterodimerizing with SMAD4. This complex then translocates to the nucleus to regulate transcription.

Phosphorylation of the Glucocorticoid Receptor Alters SMAD Signaling in Vocal Fold Fibroblasts

Shigeyuki Mukudai, MD, PhD; Nao Hiwatashi, MD, PhD; Renjie Bing, MD; Michael Garabedian, PhD; Ryan C. Branski, PhD ©

Objectives/Hypothesis: Direct glucocorticoid (GC) injection for vocal fold (VF) scarring has evolved as a therapeutic strategy, but the mechanisms underlying the antifibrotic effects remain unclear. GCs act via the glucocorticoid receptor (GR), which is phosphorylated at multiple serine residues in a hormone-dependent manner to affect bioactivity. We hypothesize that GCs regulate SMAD signaling via GR phosphorylation in vocal fold fibroblasts (VFFs).

Study Design: In vitro.

Methods: Human VFFs were treated with dexamethasone (DM; 10^{-5}–10^{-7}M) ± transforming growth factor (TGF)-β1 (10 ng/mL). RU486 (10^{-6}M) was employed to isolate the regulatory effects of GR. Total GR, Ser211, and Ser203 phosphorylation was examined via sodium dodecyl sulfate-polyacrylamide gel electrophoresis and immunocytochemistry. Quantitative polymerase chain reaction was employed to determine GR-mediated effects of DM on genes related to fibrosis.

Results: Total GR and Ser211 phosphorylation was observed predominantly in the nucleus 1 hour after DM administration. DM decreased total GR expression, but Ser203 and Ser211 phosphorylation increased. RU486 limited the effects of DM. SMAD3 and SMAD7 mRNA expression significantly decreased 4 hours after DM administration (P < 0.05); this response was negated by RU486. COL1A1 remained unchanged, and ACTA2 significantly increased following 24 hours of DM treatment (P < 0.05).

Conclusion: DM regulated TGF-β1 signaling via altered SMAD3 and SMAD7 expression. This response was associated with altered GR phosphorylation. These findings provide insight into the mechanisms of steroidal effects on vocal fold repair; ultimately, we seek to enhance therapeutic strategies for these challenging patients.

Key Words: Vocal fold, voice, glucocorticoid, glucocorticoid receptor, phosphorylation, transforming growth factor-β.

Level of Evidence: NA

Laryngoscope, 129:E187–E193, 2019

DOI. 10.1002/lary.27570

From the Department of Otolaryngology–Head and Neck Surgery (S.M., N.H., R.B., R.C.B.), NYU Voice Center; the Department of Microbiology (M.G.), New York University School of Medicine, New York, New York, U.S.A.

Editor's Note: This Manuscript was accepted for publication on August 20, 2018.

Portions of these data were accepted for presentation at the American Laryngological Association, Combined Otolaryngology Spring Meetings, National Harbor, Maryland, U.S.A., April 18–20, 2018.

Funding for this work was provided by the National Institute on Deafness and Other Communication Disorders (NICD/the National Institutes of Health (NIH), R01 DC012977 and R01 DC017397, PI: Branski). The authors have no other funding, financial relationships, or conflicts of interest to disclose.

Send correspondence to Ryan C. Branski, PhD, NYU Voice Center, Department of Otolaryngology-Head and Neck Surgery, New York University School of Medicine, 345 East 37th Street, Suite 306, New York, NY 10016. E-mail: ryan.branski@nyumc.org

Laryngoscope 129: May 2019 Mukudai et al.: Steroids Alter Smad Signaling
Conversely, SMAD7 is a competitive inhibitor of SMAD activation, and therefore is thought to be antifibrotic. Activated Smads have been implicated in a variety of fibrotic processes, suggesting that Smad activation plays a central role in fibrosis.9 Ideally, therapies for vocal fold fibrosis should likely alter the fibroblast phenotype, and emerging data suggest that GCs attenuate TGF-β/SMAD signaling in human fetal lung fibroblasts.10,11 Fetal lung fibroblasts are likely a unique niche; we sought to investigate the effects of GCs on vocal fold fibroblasts (VFFs).

Emerging evidence suggests that fibroblasts are target cells for GCs and express the glucocorticoid receptor (GR).12–14 Our laboratory recently immunolocalized the GR in the vocal fold mucosa and GCs altered VFF cell growth, the expression of ECM genes, and collagen secretion.12,14 GCs pass through the cell membrane and form complexes by binding with nuclear receptors. The resulting complex binds to the DNA of specific genes and regulates transcription. Although ligand binding is essential for activation of GR, the receptor is also subject to post-translational modification through phosphorylation.15 GR is phosphorylated in the absence of hormones, and additional phosphorylation events may occur following agonist binding. Three major serine sites are phosphorylated within the N-terminal region of the receptor and are involved in transcriptional regulation (Ser203, Ser211, and Ser226). The specific GR phosphorylation site may determine target gene activity, cofactor interaction, strength and duration of receptor signaling, and/or or receptor stability. For example, phosphorylation at Ser211 has been associated with nuclear translocation and transcriptional activation of GR after hormone treatment. Phosphorylation at Ser203 has been shown to be a determinant of ligand-dependent downregulation of GR.16,17 Novel phosphorylation-specific antibodies to these phosphoserine sites were recently developed to allow for analysis of GR phospho-isofor expression and localization with increased fidelity.16,17 We hypothesized that GCs regulate Smad signaling via GR phosphorylation in VFFs. Therefore, we sought to quantify the effects of dexamethasone (DM) on Ser211 and Ser203 phosphorylation and regulation of TGF-β1 signaling.

MATERIALS AND METHODS

Cell Culture
An immortalized human vocal fold fibroblast cell line created in our laboratory was employed for all experimentation. This cell line, referred to as HVOX, has been shown to be stable through multiple population doublings; cells in passages 20 to 30 were used. Cells were cultured on plates in phenol red-free Dulbecco’s Modified Eagle’s medium (DMEM) containing 10% charcoal–dextran–treated fetal bovine serum and 1% antibiotic/antimycotic (Life Technologies, Grand Island, NY) at 37°C under standard cell culture conditions.

Immunocytochemistry for GR
HVOX were seeded on chamber slides for 2 days and examined after 1 hour of incubation at 37°C in the presence of 10−7 M DM (Sigma-Aldrich, St. Louis, MO) or control (equal volume of dimethyl sulfoxide). All cells were fixed with 4% paraformaldehyde at 37°C for 10 minutes, washed with phosphate-buffered solution (PBS), and blocked for 1 hour at room temperature with PBS solution containing 0.3% Triton-X and 5% bovine serum albumin. Cells were then incubated with the following antibodies for 24 hours at 4°C: rabbit anti-total GR (1:100; Cell Signaling, Danvers, MA); rabbit anti-hGR-S203 (1:500); and rabbit anti-hGR-S211 (1:1000). Alexa-Fluor 488 goat anti-rabbit IgG (1:500; Invitrogen, Carlsbad, CA) were used as secondary antibody. Stained sections were visualized, and images were captured using a Nikon Eclipse Ni-U fluorescence microscope (Nikon Inc., Tokyo, Japan).

Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis
Following 12 hours of serum starvation, HVOX were treated with serum/phenol red-free DMEM ± DM (10−7 M) ± TGF-β1 (10 ng/mL) ± RU486 (10−6 M, Sigma-Aldrich) and harvested at 1 hour, or with serum/phenol red-free DMEM ± DM (10−7 M) and harvested at 1, 4, 24, or 48 hours. Recombinant human TGF-β1 was employed for experimentation. RU486, a GR antagonist, was employed to isolate the regulatory effects of GR. After treatment, the chamber slide inserts were removed, and the plates were washed twice with cold PBS. HVOXs were harvested via cell scraping, and total cellular protein was extracted using the Mammalian Protein Extraction Reagent (Thermo Scientific, Waltham, MA), supplemented with Halt Protease Inhibitor Cocktail (Thermo Scientific), 0.5M EDTA Solution 100 x (Thermo Scientific), Calyculin A (Cell Signaling), and 2-mercaptoethanol (Life Technologies). Total protein was quantified via the Pierce 660nm Protein Assay (Thermo Scientific). Each protein lysate was loaded on 8% sodium dodecyl sulfate-polyacrylamide gels. Protein was then transferred to PVDF membranes (Invitrogen) and blocked with 1-Block (Applied Biosystems, Foster City, CA) overnight at 4°C. Membranes were incubated with a primary antibody against rabbit anti-total GR (1:1000; Cell Signaling) for 1 hour, rabbit anti-hGR-S203 (1:3000) for 3 hours, rabbit anti-hGR-S211 (1:1000) overnight, or β-actin (1:5000; Cell Signaling) for 1 hour at 4°C, followed by 1-hour incubation with horseradish peroxidase-conjugated secondary antibodies (Cell Signaling). Blots were visualized using chemiluminescence detection.

Quantitative Real-Time Polymerase Chain Reaction
Following 12 hours of serum starvation, cells were treated with serum/phenol red-free DMEM ± DM (10−5, 10−6, 10−7 M, Sigma-Aldrich) ± TGF-β1 (10 ng/mL, Life Technologies) ± RU486 (10−6 M, Sigma-Aldrich) and harvested at 4 and 24 hours. Total RNA was extracted via the RNeasy Mini Kit (Qiagen, Valencia, CA) and reverse transcribed with a High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems). The TaqMan Gene Expression kit (Life Technologies) and StepOne Plus (Applied Biosystems) were employed for quantitative analyses. Tagman primer probes for SMAD3 (Hs00969210_m1), SMAD7 (Hs0098193_m1), COL1A1 (Hs00164004_m1), ACTA2 (Hs00426835_g1), and GAPDH (Hs02758991_g1) were employed. The ΔΔCt method was employed with GAPDH as the housekeeping gene for the determination of relative expression levels.

Immunocytochemistry for ACTA2
HVOX were seeded on chamber slides for 2 days. Following 12 hours of serum starvation, cells were treated with serum/
phenol red-free DMEM ± DM (10⁻⁷ M, Sigma-Aldrich) ± TGF-β1 (10 ng/mL, Life Technologies) and harvested at 48 hours. All cells were fixed with 4% paraformaldehyde at 37°C for 10 minutes, washed with PBS, and blocked for 1 hour at room temperature with PBS solution containing 0.3% Triton-X and 5% bovine serum albumin. The slides were incubated at 4°C overnight with primary mouse monoclonal antibodies against ACTA2 (1:100; Sigma-Aldrich) and then with the corresponding Alexa-Fluor 555 goat anti-mouse immunoglobulin G (1:500; Invitrogen) secondary antibody. Nuclei were counterstained with DAPI. Digital images were captured, and both the total cell count and ACTA2 positive cells were counted in 10 randomly chosen, 3.0 mm² fields for each condition under 10 × magnification.¹⁸

**Statistical Analyses**

Data were expressed as means ± standard error of the mean and analyzed via one-way analysis of variance. If a main effect was observed, post hoc Tukey tests for data with normal distribution and Kruskal-Wallis and Mann-Whitney U tests for data with non-normal distribution were performed using SPSS Statistics for Windows version 23 (IBM Corp., Armonk, NY). P < 0.05 was considered significant.

**RESULTS**

**Subcellular Distribution of Endogenous Ser²⁰³ and Ser²¹¹ Phosphorylated Forms of GR**

In the absence of DM, total GR and Ser211 immunoreactivity were detected in the nucleus and cytoplasm of HVOX. Following DM exposure, localization shifted predominantly to the nucleus (Fig. 1A, 1B). Ser203 phosphorylation was localized predominantly in the cytoplasm in the absence and presence of DM (Fig. 1C).

**Effects of DM, RU486, and TGF-β1 on GR Phosphorylation**

As shown in Figure 2, whereas DM (10⁻⁷ M) decreased total GR expression, Ser²⁰³ and Ser²¹¹ phosphorylation increased. RU486 (10⁻⁶ M) limited the effects of DM. TGF-β1 (10 ng/mL) did not alter total GR expression or phosphorylation of Ser²⁰³ and Ser²¹¹.

**DM Downregulated Total GR**

DM (10⁻⁷ M) reduced total GR expression in a time-dependent manner (Fig. 3).

**DM Altered Gene Expression of SMAD3, SMAD7, COL1A1, and ACTA2**

SMAD3 and SMAD7 significantly decreased 4 hours after DM exposure (Fig. 4A and 4B). COL1A1 remained unchanged, and ACTA2 significantly increased following 24 hours of DM treatment (Fig. 4C and 4D). No concentration effect for DM was noted. RU486 reduced DM-induced decrease in SMAD3 and SMAD7 mRNA expression (Fig. 5A and 5B). RU486 reduced DM-induced increase in ACTA2 mRNA expression (Fig. 5C).

---

**DM Altered TGF-β1-Mediated Transcriptional Events**

TGF-β1 significantly increased SMAD7, COL1A1, and ACTA2 mRNA expression (Fig. 6B, 6C, and 6D). The combination of DM and TGF-β1 significantly decreased SMAD3 and SMAD7 (Fig. 6A and 6B) compared to TGF-β1 alone at 4 hours. COL1A1 was unchanged (Fig. 6C),
and ACTA2 significantly increased compared to TGF-β1 alone (Fig. 6D) following 24 hours of DM treatment.

**DISCUSSION**

Effective therapeutics and preventative strategies for vocal fold scar remain elusive. We hypothesize that enhanced understanding of key biochemical triggers underlying the sequence of events leading to the development of the aberrant, fibrotic phenotype is likely to elucidate novel targets for intervention. Glucocorticoids have historically been thought to be powerful immune modulators with ubiquitous use across inflammatory processes. The utility of GCs in more chronic fibroplastic processes is less clear. However, emerging clinical data suggest some degree of efficacy of direct steroid injection into vocal fold scar.4 We sought to investigate potential interactions between glucocorticoids and TGF-β, a mediator of fibrosis across tissues including the vocal folds.

In the current study, DM regulated GR phosphorylation and TGF-β1 signaling in human VFFs. To the best of our knowledge, these data are the first to describe immunoreactivity and changes in specific GR residues (203 and 211) associated with GC exposure in VFFs. Moreover, DM decreased SMAD3 mRNA expression consistent with its anti-fibrotic potential. However, DM also increased TGF-β1-induced fibroblast-myoﬁbroblast differentiation, which is inconsistent with the concept of glucocorticoids holding signiﬁcant antiﬁbrotic potential.
The distribution of total GR in HVOX indicated that ligand binding led to near complete nuclear localization of the receptor, which is consistent with our previous reports. DM acted on human VFFs directly via GR. Similarly, the distribution of Ser203 and Ser211 phosphorylation was also consistent with our previous report, suggesting that differentially phosphorylated receptor residues were located in unique subcellular compartments, likely modulating distinct aspects of receptor function. DM downregulated total GR over time and increased Ser203 and Ser211 phosphorylation in human VFFs. These effects were extinguished with RU486, a GR antagonist. These findings are largely consistent with established dogma. However, we conceptualized a model of GR modulation via phosphorylation in the absence of DM. Although DM stimulated GR phosphorylation primarily at Ser203, a population of unphosphorylated receptor molecules may persist. With time, Ser203 underwent dephosphorylation such that the ratio of phosphorylated Ser211 to phosphorylated Ser203 increased. Differential modification of GR by phosphorylation likely induces a distinct conformation and influences GR association with additional coregulatory proteins to ultimately modulate GR transactivation, stability, and subcellular location.

Dexamethasone significantly decreased SMAD3 and SMAD7, and increased ACTA2 mRNA in our cell line. Previous reports suggested that GR inhibited TGF-β signaling by directly targeting the transcriptional activation function of SMAD3, which is consistent with our data. Our laboratory and others identified SMAD3 as a principal mediator of fibrotic VF wound healing due to its role in TGF-β signaling, which in turn regulates numerous fibroblast activities including migration, proliferation, and production of extracellular matrix.

Fig. 6. SMAD3 (A), SMAD7 (B), COL1A1 (C), and ACTA2 (D) mRNA levels were examined by quantitative real-time polymerase chain reaction 4 or 24 hours after TGF-β1 ± DM. n = 5 for all; *P < 0.05 versus control; Φ P < 0.05 versus TGF-β1.DM = dexamethasone; TGF = transforming growth factor.

Fig. 7. Effects of DM on ACTA2 expression. (A) Representative immunofluorescent images of human vocal fold fibroblasts under experimental conditions. Blue, DAPI and red, ACTA2. Scale bar: 100 μm. (B) Ratio of ACTA2-positive cells was calculated from 10 randomly chosen fields of four samples in each group. The ratio of ACTA2-positive cells under TGF-β1 stimulation was significantly increased 48 hours after DM administration. N = 5; *P < 0.05 versus control; #P < 0.05 versus DM; Φ P < 0.05 versus TGF-β1.DAPI = 4', 6-Diamidino-2-phenylindole dihydrochloride; DM = dexamethasone; TGF = transforming growth factor.
these data suggest that DM has antifibrotic effects. However, SMAD7, a homolog and competitive inhibitor of SMAD3, was also downregulated. SMAD7 downregulation in response to DM was both contradictory to our hypothesis and conceptually interesting. One possible explanation is that this response may be a component of a feedback loop for SMAD3 downregulation.

Collectively, these responses were negated by RU486, suggesting GR dependence. Interestingly, GCs were previously shown to have no effect on SMAD expression in human fetal lung fibroblasts, mouse and rat osteoblastic cells, and human trabecular meshwork cells and tissues, suggesting some geographic specificity. In addition, no concentration effect evolved with regard to DM exposure in our cells. Unfortunately, these data provide little insight regarding relevant concentrations for clinical use, and this issue warrants further investigation. DM did not decrease COLIA1 mRNA in human VFFs, which is contradictory to our previous work regarding the effects of DM on collagen secretion. This discrepancy, however, is consistent with data from other cells. The mechanism was thought to be lysosome-mediated autophagy-induced degradation of COLIA1 protein abundance by cortisol. These phenomena warrant further investigation.

In addition, DM increased ACTA2 mRNA and the ratio of ACTA2 positive cells in our cell line, suggesting DM synergized with TGF-β1 to induce fibroblast–myofibroblast differentiation consistent with data from human fetal lung fibroblasts. These data are wholly contrary to our overarching hypothesis and may underlie suboptimal outcomes with DM treatment in the clinical milieu. For example, no differences were reported between steroid-injected and control groups after vocal fold surgery. Furthermore, prior data did not support the use of DM to prevent pulmonary fibrosis, and the authors speculated that DM actually enhanced the fibrotic response. GCs also adversely affected patient prognosis in idiopathic pulmonary fibrosis. In addition, with regard to mechanism, previous work suggested that prednisolone suppressed lung inflammatory edema but failed to suppress pulmonary fibrosis. These results suggest that GCs may effectively limit the acute inflammatory response following vocal fold injury but are less ideal for more chronic vocal fold fibrosis. Clearly, the timing of GC administration may be of critical importance to effectuate therapeutic efficacy; further investigation in this regard is warranted. Furthermore, the current study is not without limitation. As noted previously, GCs are a vast category of compounds with diverse pharmacokinetic profiles. The current study included only DM because it appears to hold the highest therapeutic (e.g., half-life, anti-inflammatory potential). It is unclear if these effects are consistent across GCs. With the evolution GC use in laryngology, further investigation is fundamental to optimal therapeutics.

CONCLUSION

DM-regulated TGF-β1 signaling via altered SMAD3 and SMAD7 expression. This response was associated with altered GR phosphorylation. These findings provide preliminary insight regarding the mechanisms of steroidal effects on vocal fold injury, with the goal of enhanced therapeutic strategies for these challenging patients.

BIBLIOGRAPHY


