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Inhibitory effects of HGF and IL-6 on TGF- β 1 mediated vocal fibroblast-myofibroblast differentiation

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Abstract

The role of myofibroblasts in vocal fold scarring has not been extensively studied partly due to a lack of a robust *in vitro* model. The objective of this investigation was to develop and characterize a myofibroblast *in vitro* model that could be utilized to investigate the molecular mechanism of myofibroblast differentiation and function in injured vocal fold tissue. Differentiation of human primary vocal fold fibroblasts (hVFF) to myofibroblasts was stimulated using 5, 10, or 20 ng/ml of recombinant transforming growth factor beta-1 (TGF- β 1). Cultures were analyzed using immunofluorescence and western blotting, with an anti-alpha smooth muscle actin (α -SMA) antibody as a myofibroblast marker. Normal rabbit vocal folds were treated with 10 ng/ml of TGF- β 1 for 7 days for *in vivo* corroboration. The effects of interleukin-6 (IL-6) and hepatocyte growth factor (HGF) on myofibroblast differentiation were studied using western blots. hVFF demonstrated positive α -SMA labeling in 10 and 20 ng/ml TGF- β 1 stimulated cells indicating that hVFFs were capable of differentiation to myofibroblasts. TGF- β 1 induced the largest increase in α -SMA at 10-ng/ml on day 5 of treatment. HGF and IL6 suppressed the expression of TGF- β 1 induced α -SMA. Our work characterizes a useful *in vitro* model of TGF- β 1 mediated vocal fibroblast-myofibroblast differentiation. The extent of differentiation appears to be attenuated by HGF suggesting a potential mechanism to support prior work indicating that HGF plays a protective role from scar formation in vocal fold injuries. Paradoxically, IL-6 which has been shown to play a profibrotic role in dermal studies also attenuated the TGF- β 1 response.

Keywords

fibroblasts; TGF- β 1; α -SMA; vocal fold; myofibroblasts; wound healing

Introduction

Vocal fold scarring results from numerous pathological and non-pathological conditions and has an adverse affect on the functionally critical biomechanical properties of this tissue's lamina propria(1). The altered physical characteristics of the scarred vocal fold can result in persistent dysphonias that are difficult to treat. An increasing body of research continues to study the physical, histological, and biochemical mechanisms of vocal fold healing and scarring in an attempt develop strategies to treat vocal fold injuries prior to scar formation. Wound studies in other systems have found that one of the early steps in wound healing involves the recruitment of fibroblasts to the site of injury. Fibroblasts play an important role in generating a mature extracellular matrix (ECM) containing collagen and are regulated by inflammatory cells, epithelial cells, and other fibroblasts.

Fibroblasts are central to the healing and scarring processes; they have been found to have distinct phenotypes depending upon their site of isolation(2). It has been observed that as wound repair proceeds, fibroblasts become activated and undergo a phenotypic transition expressing α -smooth muscle actin (α -SMA)(3,4). These modified fibroblasts have been classified as myofibroblasts. Functionally, myofibroblasts are thought to play a role in wound contracture, closure and the formation of collagen rich scar. The presence of myofibroblasts in tissues has been established as a marker of progressive fibrosis(4). While myofibroblasts play an important role in the earlier stages of wound healing, it has been found that their numbers normally decrease as the wound is closed. Electron microscopy(5) and DNA labeling studies(6) have revealed that the myofibroblast numbers decrease in a concerted apoptotic manner. Any disruption to this concerted behavior has been found to result in excess ECM deposition and subsequent scar formation. Currently, the role of myofibroblasts in vocal fold scarring has not been extensively studied partly due to a lack of a robust *in vitro* model of vocal fold myofibroblasts. Such models have been used successfully to provide insight into fibrogenesis and suggest novel strategies for modulation of wound healing in the lung(7,8), eye(9), and liver(10). The goal of this study is to develop and characterize a myofibroblast cell culture model that could be utilized to investigate and understand the molecular mechanism of myofibroblast differentiation and function in injured vocal fold tissue. We further wish to examine the effect of hepatocyte growth factor (HGF) and interleukin 6 (IL-6) on the development of the myofibroblast phenotype. HGF is a potent mediator of cellular proliferation, migration, survival and tissue regeneration. HGF and its receptor, c-met, have been found in the vocal fold. A number of studies have suggested that HGF plays a protective role in vocal fold fibrosis and scarring. However, the mechanisms responsible for HGF dependent inhibition of vocal fold fibrosis are poorly understood. IL-6 is a pleiotropic cytokine whose role in wound closure is poorly understood. It has been demonstrated that IL-6 can modulate α -SMA in primary skin fibroblast cultures, implicating the role of IL-6 in the development of treatment for wounds. It is unknown if this is the same for the vocal fold.

Materials and Methods

Differentiation to myofibroblasts was stimulated using 5, 10, or 20 ng/ml of recombinant transforming growth factor beta-1 (TGF- β 1). Cultures were analyzed using immunofluorescence and western blotting with an anti-alpha smooth muscle actin (α -SMA) antibody as a myofibroblast marker. Additionally, *in vivo* stimulation of normal vocal fold

lamina propria with recombinant TGF- β 1 was analyzed with western blotting to verify the *in vitro* model. We further examined the effect of hepatocyte growth factor (HGF) and interleukin 6 (IL-6) on the development of the myofibroblast phenotype.

Vocal fold lamina propria fibroblast isolation and culture

Normal human vocal fold tissue obtained from a 59 year-old female donor, whose vocal fold was judged to be normal without any evidence of disease by the attending surgeon, and the donor did not have a history of smoking or laryngeal surgery. Tissue was resected and immediately placed in sterile PBS. The research protocol was conducted with approval from the Institutional Review Board of University of Wisconsin-Madison. True vocal fold tissue (epithelium and lamina propria) was cut into small pieces and suspended in DMEM supplemented with 10% fetal bovine serum (FBS), 100 U/mL penicillin, 0.01 mg/mL streptomycin sulfate and 1 $\times$ NEAA (all from Sigma, St Louis, CA). Cells were grown on uncoated plastic tissue culture dishes (Focal) at 37°C in 5% CO₂-humidified atmosphere. After 14 days, the adherent confluent human vocal fold fibroblasts (hVFF) were trypsinized, passaged. The fibroblast categorization and identification has previously been reported with this culture methodology(11). All experiments were performed on cells that ranged between passages 4 through 9.

Immunofluorescent cell staining

Cell morphology was studied using immunofluorescent staining with antibodies directed against alpha-smooth muscle actin (α -SMA) and vimentin (all from Sigma, St Louis, MO). Myofibroblasts were defined by the presence of α -SMA. The hVFFs were seeded into sterile Permanox 8-chamber slides (Nunc, Thermo Fisher Scientific, Rochester, NY) at a density of 2 $\times$ 10⁴ cells per cm² in DMEM-10% FBS and incubated until they reached 50% confluence which typically occurred in 4 days. Cells were then washed with sterile PBS and treated with either serum-free DMEM or serum-free DMEM with 10 ng/mL or 20 ng/mL TGF- β 1 (Sigma, St Louis, MO). Following 5 days of TGF- β 1 treatment, the cells were washed three times with ice-cold PBS for 10 minutes each. The cells were fixed with 4% paraformaldehyde for 15 minutes at room temperature. All steps following fixation were performed at room temperature. Following fixation, cells were washed three times with PBS for 10 minutes each. The cells were permeabilized for 60 minutes in 0.5% Triton X-100 (Sigma, St Louis, MO) and 10% FBS in PBS for α -SMA staining or 10% normal rabbit serum in PBS for vimentin staining within a humidified chamber. The primary antibody was diluted to the appropriate concentration (mouse anti- α -SMA at 1:400 and goat anti-vimentin at 1:20) in PBS with 0.3% Triton X-100 and 2% FBS. The cells were incubated in the primary antibody for 60 minutes at room temperature in a humidified chamber. Each slide had a serum-treated and TGF- β 1 treated negative control in which the cells were incubated with antibody dilution solution without primary antibody during this time. The cells were then washed three times with the same solution used to dilute the respective antibody for 10 minutes each. The species-specific secondary antibodies were diluted to the appropriate concentration [FITC-labeled rabbit anti-mouse IgG (Sigma, St Louis, MO) at 1:200 and rhodamine-labeled rabbit anti-goat IgG (Jackson ImmunoResearch Laboratories, West Grove, PA) at 1:100] in PBS with 0.3% Triton X-100 and 2% FBS for α -SMA staining or 2% normal rabbit serum for vimentin staining. The cells were incubated in the secondary antibody for 30 minutes at room temperature in a dark humidified chamber. The cells were then washed three times with the same solution used to dilute the respective secondary antibody for 10 minutes each. The chambers and gaskets were carefully removed and the slides were mounted with VECTASHIELD Mounting Medium. Mounted slides were immediately examined on a Nikon Eclipse E600 fluorescent microscope (Nikon, Melville, NY) and images were captured on a Pixera color camera (Pixera, Los Gatos, CA).

Preparation of cell culture lysates for western blot analysis

HVFFs were seeded into sterile uncoated 6-well plates at a density of 2×10^4 cells per cm^2 in DMEM with 10% FBS and were grown in a 37°C incubator with 5% CO_2 . Dose-response experiments were initiated when cultures reached 50% confluence which typically occurred in 4 days. Cells were washed with sterile PBS and treated with either serum-free DMEM or serum-free DMEM with 0, 5 ng/mL, or 10 ng/mL TGF- β 1 for 7 days. For the time-course experiment, the hVFFs were treated with serum-free DMEM with 10 ng/mL TGF- β 1 for 1, 3, 5 and 7-day time points. At day 4, fresh media with TGF- β 1 were changed. For HGF and IL-6 experiments, the cells were starved with DMEM containing 0.1% BSA for 24 hours at the point they reached 70-80% confluency. After the starvation period, the cells were treated with TGF- β 1 (10 ng/mL) and HGF (0, 5, 10, 20, or 40 ng/mL) or IL-6 (0, 5, 10, 20, or 40 ng/mL) for 24 hours. At the end of incubation, the cells were washed with PBS and the total cell protein was extracted by using the M-PER protein extraction kit (Pierce, Rockford, IL) following the manufacturer's instructions. Protein was quantified using a BCA assay method (Pierce, Rockford, IL). The protein aliquots were stored at -80°C .

In vivo model

Twelve New Zealand white breeder rabbits were used in this study, which was approved by the University of Wisconsin Madison Institutional Animal Care and Use Committee. Rabbits were anesthetized with an intravenous injection of Xylazine 35 mg/kg, Ketamine 5 mg/kg and Acepromazine 0.75 mg/kg and were ventilated with supplemental O_2 . The larynges were visualized with a Pilling infant Hollinger pediatric endoscope (Pilling Horsham, Storz, Culver City, CA) as previously described(12). Left vocal folds were injected with two 0.1 mL of recombinant TGF- β 1 (10 ng) injections – one in the medial edge and the other at the posterior end of medial edge using a 26-gauge needle (Microfrance, Terrebonne, QC, Canada). The right vocal fold margins were injected with two 0.1 mL injections of PBS as control. Immediately after surgery, Buprenex (0.05 mg/kg) was provided for pain management. The above procedures were completed daily for 4 time points – 1, 3, 5 and 7 days. At each of these time points, 3 rabbits were sacrificed by IV administration of Beuthanasia-D (390 mg/mL). All vocal folds were removed from the larynges and were immediately kept in RNAlater (Ambion, Austin, Texas). After 24 hours, the vocal fold tissues were transferred into -80°C for storage.

Preparation of tissue lysates for western blot

Frozen vocal fold tissue samples were thawed and minced quickly with a razor blade, then placed in ice-cold T-PER Tissue Protein Extraction Reagent (Pierce, Rockford, IL) with protease inhibitors in a ice-bath, and homogenized immediately using a homogenizer (PRO Scientific, Oxford, CT). The lysates were centrifuged at 14,000 rpm for 10 min at 4°C , and the supernatants were transferred, aliquoted and kept at -80°C until analysis. Total protein was measured by BCA assay as described above.

Western blot

For each protein sample, 1.5 μg of total protein was characterized electrophoretically under reducing conditions on precast NuPAGE 10% Bis-Tris gels (Invitrogen, Carlsbad, CA) using NuPAGE MOPS SDS buffer and transferred onto nitrocellulose membrane using an XCELL II Blot Module (Invitrogen, Carlsbad, CA). The MagicMark XP western protein standard (Invitrogen, Carlsbad, CA) was used to validate bands in the range of 20–220 kDa. Transfer efficiency was confirmed by staining blot membranes with Ponceau S stain (Sigma, St Louis, MO). The Ponceau S stain was rinsed from the membrane and the membranes were probed for α -SMA using an anti α -SMA antibody produced in mouse clone 1A4 (Sigma, St Louis, MO) and GAPDH using a mouse anti-GAPDH antibody (Sigma, St Louis,

MO) as a loading control. Briefly, the membranes were blocked for 1 hour in 5% Carnation dry milk powder in Tris-buffered saline at room temperature or overnight at 4°C. The membrane was then incubated with a 1:20,000 dilution of anti-GAPDH antibody and a 1:1,000 dilution of a mouse monoclonal anti- α -SMA monoclonal antibody for 1 hour at room temperature. Bound antibodies on the nitrocellulose membrane were detected using the WesternBreeze anti-mouse chemiluminescent kit (Invitrogen, Carlsbad, CA). The chemiluminescent signal was transferred to film and scanned using Adobe Photoshop 7. Densities of the bands were measured using ImageJ (NIH), and plotted as a ratio using Microsoft Excel and SigmaPlot.

Results

TGF- β 1 induces α -SMA expression in hVFF

To characterize differentiation of the myofibroblast phenotype in hVFF, we treated hVFF at various dosages and times with TGF- β 1. Phenotypic conversion of the hVFF was detected by α -SMA immunofluorescence (Figure 1) and western blot analysis (Figure 2). Immunofluorescence indicated cells with a transformed morphology showing hypertrophy, elongated morphology and strong α -SMA staining in cytoplasmic myofilaments along the cell axis. Dose-dependent treatment with TGF- β 1 upregulated α -SMA in a linear manner. Whereas time-dependent treatment with 10ng/ml TGF- β 1 (Figure 2) upregulated α -SMA up to day 5, after which a decline in α -SMA was measured.

TGF- β 1 induces α -SMA expression in vocal folds *in vivo*

We next explored if a similar TGF- β 1 induced increase in α -SMA expression could be measured *in vivo*. Rabbit vocal folds were treated with unilateral injections of TGF- β 1 (10 ng/ml) over a 7 day period to determine if there was an induction of fibroblast-myofibroblast differentiation. For control, saline was injected into the contralateral vocal folds. Tissues were removed, homogenized, protein extracted and assayed for α -SMA protein expression with immunoblotting. Over a 7 day period there was a gradual upregulation of α -SMA (Figure 3). There was a concomitant upregulation of α -SMA in the saline treated vocal fold.

HGF and IL-6 inhibits TGF- β 1 mediated fibroblast-myofibroblast differentiation

HGF modulates cell motility, morphogenesis, survival and proliferation. These effects may play an important role in the protective action of HGF in models of vocal fold scarring *in vivo*, as demonstrated by the literature. We examined whether HGF could alter levels of α -SMA protein in hVFF cells. Cells were exposed to increasing concentrations (5-40 ng/ml) of HGF for 24 hours in the presence of TGF- β 1, then lysed and assayed for α -SMA protein expression by immunoblotting. While lower concentrations of HGF did not alter TGF- β 1 induced α -SMA expression, concentrations of 20 and 40 ng/ml inhibited expression of the protein (Figure 4).

IL-6 has been shown to modulate TGF- β 1 dependent α -SMA expression in skin fibroblasts. This phenomenon may be relevant in hVFF, as such we examined whether IL-6 could alter levels of α -SMA protein in hVFF cells. Cells were exposed to increasing concentrations (5-40 ng/ml) of IL-6 for 24 hours in the presence of TGF- β 1, then lysed and assayed for α -SMA protein expression by immunoblotting. All concentrations of IL-6 inhibited TGF- β 1 induced α -SMA (Figure 5).

Discussion

In this study, we have shown that isolated human vocal fold fibroblasts (hVFF) have the TGF- β 1 dose-dependent ability to differentiate to a myofibroblast phenotype that expresses α -SMA. A similar effect was measured in our *in vivo* rabbit model. Furthermore, the extent of differentiation appears to be attenuated by HGF suggesting a potential mechanism to support prior work indicating that HGF plays a protective role from scar formation in vocal fold injuries. Paradoxically, IL-6 which has been shown to play a pro-fibrotic role in dermal studies also attenuated the TGF- β 1 response. The myofibroblast culture model characterized in this study will be useful in future studies that seek to elucidate the mechanisms of potential therapeutics targeting the reduction or prevention of vocal fold fibrosis.

Vocal fold tissue is composed of layered epithelium, lamina propria and muscle. Fibrosis of this tissue has been characterized in various animal models as aberrant fibroblastic activity specifically in the lamina propria(13,14). Scarring of the vocal folds has been characterized by changes in levels of collagen, elastin, hyaluronic acid, fibronectin, fibromodulin, decorin and others(13-17). Recently a growing body of contradictory literature has implicated TGF- β 1 in vocal fold fibrosis(18). Elevated TGF- β 1 levels have been measured in rat vocal folds 8 – 72 hours following acute injury(18). However, another rat injury study indicated that elevated TGF- β 1 levels were not measured until day 7 post injury(19). In rabbit vocal folds treated with hydrogels at the time of injury, elevated TGF- β 1 was measured on days 3 and 5 post injury(12). Most recently the effects of TGF- β 1 on vocal fold fibroblasts on collagen synthesis, migration and Smad mRNA expression has been reported by Branski et al.(20). Even though the effects of TGF- β 1 are well known in other systems, the effects of this potent mediator of wound healing is poorly understood in the vocal fold. These disparate findings in the literature to date underscore the need for an *in vitro* model to provide insight into the molecular mechanism of myofibroblast differentiation and function in injured vocal fold tissue.

In our study we were able to demonstrate a dose-dependent effect of TGF- β 1 that is consistent with that reported in the literature with other fibroblast types(21,22). We also report a time-dependent TGF- β 1 effect within the first 5 days of culture. Others have previously shown a similar effect, however with the induction of increased α -SMA for longer periods of culture(21). Our decrease in α -SMA expression at day 7 may have been due to the effects of extended serum-free growth conditions or deteriorating cell culture conditions resulting from an extended period without a change in medium.

Positive presence of α -SMA was noted only in both our TGF- β 1 and saline treated vocal folds in a time-dependent manner through the 7 days of treatment. In the former situation, higher levels of α -SMA were measured by immunoblotting. However, in the latter situation, positive staining of α -SMA was unexpected and may have been an indication of repeated injury secondary to daily injections.

HGF suppresses expression of TGF- β 1 and aids in the degradation of ECM components lending to anti-fibrogenic properties. Our findings are consistent such that we had suppression of the myofibroblast phenotype with TGF- β 1 (20-40 ng/ml). Furthermore these findings corroborate reported decreases in TGF- β 1 levels in injured vocal fold tissue treated with HGF, combined with decreased fibrosis, scarring and improved function of vocal tissues treated with HGF(19,23,24). Specific changes in the magnitude of fibroblast-myofibroblast differentiation as measured by α -SMA levels has yet to be measured *in vivo* with HGF treatment.

IL-6 is a proinflammatory cytokine that plays an important role in wound healing. It has been shown to be strongly upregulated during the inflammatory phase of healing. A

complete lack of IL-6 causes impaired wound healing and excessive amounts have been associated with cutaneous scarring(25,26). Increases in IL-6 expression after injury is transient in fetal wounds but prolonged in adult wounds(27). IL-6 administered to fetal wound for a prolonged period result in scar formation. In this study the presence of IL-6 attenuated the TGF- β 1 fibroblast-myofibroblast differentiation. Our finding supports a complex dose and time-dependent role of IL-6 in wound healing that has been suggested by studies with IL-6 knockout mice in other laboratories.

Successful development of new therapeutic interventions for vocal fold fibrosis and an improved understanding of mechanistic molecular development, pathogenesis and biological features of the vocal fold lamina propria ECM depend on the availability of robust *in vitro* models. Our preliminary work characterizes a useful *in vitro* model of TGF- β 1 mediated vocal fibroblast-myofibroblast differentiation. Further work is necessary to elucidate the complex nature of the fibroblast-myofibroblast differentiation *in vivo* to validate the model.

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Figure 1.

Human vocal fold fibroblasts (hVFF) express myofibroblast marker α -smooth muscle actin (α -SMA) when treated with TGF- β 1. VFF were plated on glass slides and treated with 20 ng/ml of TGF- β 1 for five days. Cells were fixed with 4% paraformaldehyde and immunostained for α -SMA or vimentin. The top and middle panels are representative images captured at 20 $\times$ magnification and the lower panel were captured at 100 $\times$. The top panel represents hVFF treated with or without TGF- β 1. Cells are larger in size. The middle panel represents hVFF treated with or without TGF- β 1. Vimentin (red) is used as control. The lower panel represents hVFF treated with or without TGF- β 1. α -SMA (green) is expressed in TGF- β 1 treated hVFF.

Figure 2.

Dose and time response for human vocal fold fibroblasts (hVFF) as measured by α -SMA when treated with TGF- β 1. (A.) Western blot analysis of hVFF incubated with TGF- β 1 (0, 5 or 10 ng/mL) for 7 days. Blots were analyzed by densitometry and α -SMA activity was normalized to GAPDH. The histogram is representative of three independent experiments. (B.) Western blot analysis of hVFF incubated with TGF- β 1 (10 ng/mL) for 1, 3, 5 and 7 days. Blots were analyzed by densitometry and α -SMA activity was normalized to GAPDH. The western blot is representative of three independent experiments.

Figure 3.
Time response for rabbit vocal folds treated with TGF- β 1 and saline for 1, 3, 5 and 7 days. Western blots were analyzed by densitometry and α -SMA activity was normalized to GAPDH. The histogram is representative of three independent experiments.

Figure 4.

HGF decreased TGF- β 1 induced α -SMA protein in human vocal fold fibroblasts (hVFF). HVFF were incubated with 10 ng/ml TGF- β 1 with and without HGF (5, 10, 20 and 40 ng/ml). After 24 hours, cells were lysed and samples containing equal amounts of total protein were resolved by western blots against α -SMA and GAPDH. Relative expression of α -SMA was evaluated by densitometric analysis by calculating α -SMA to GAPDH ratios. Results shown are representative of 3 independent experiments.

Figure 5.

IL-6 decreased TGF- β 1 induced α -SMA protein in human vocal fold fibroblasts (hVFF). HVFF were incubated with 10 ng/ml TGF- β 1 with and without IL-6 (5, 10, 20 and 40 ng/ml). After 24 hours, cells were lysed and samples containing equal amounts of total protein were resolved by western blot against α -SMA and GAPDH. Relative expression of α -SMA was evaluated by densitometric analysis by calculating α -SMA to GAPDH ratios. Results shown are representative of 3 independent experiments.