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Paracrine Potential of Fibroblasts Exposed to Cigarette Smoke Extract With Vascular Growth Factor Induction

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Abstract

Objectives/Hypothesis—Nicotine, a major constituent of cigarette smoke, can activate the cholinergic anti-inflammatory pathway by binding to $\alpha 7$ -nicotinic acetylcholine receptor ($\alpha 7$ nAChR) expressed on the surface of certain cells. Here, we ask whether cigarette smoke extract induced different paracrine factors compared to the in vivo regulator of inflammation, tumor necrosis factor- α , in human vocal fold fibroblasts (hVFFs) shown to express low levels of $\alpha 7$ nAChR.

Study Design—In vitro.

Methods— $\alpha 7$ nAChR was detected by nested polymerase chain reaction and immunohistochemistry. γ H2AX, a marker for DNA double-strand breaks, was measured by immunofluorescence. Cigarette smoke extract was prepared in accordance with investigators studying effects of cigarette smoke. hVFFs treated for 3 hours had media replaced for an additional 24 hours. Cytokine, chemokine, and growth factor levels in media were assessed by multiplex analysis.

Results— $\alpha 7$ nAChR expression levels decreased with the passage number of fibroblasts. Tumor necrosis factor- α induced a significantly different profile of cytokines, chemokines, and growth factor compared to cigarette smoke extract exposure. Cigarette smoke extract at a concentration not associated with induction of γ H2AX nuclear foci significantly increased vascular endothelial growth factor.

Conclusions—Cigarette smoke extract elicited a response important for regulation of angiogenesis and vascular permeability during inflammation, without evidence of DNA double-strand breaks associated with carcinogenesis. hVFFs are capable of participating in paracrine regulation of pathological blood vessel formation associated with cigarette smoking-related diseases (ie, Reinke edema). These cells express $\alpha 7$ nAChR, an essential component of the cholinergic anti-inflammatory pathway regulated by the vagus nerve in certain tissues and a target of therapeutic agents.

Keywords

Vocal fold fibroblasts; cigarette smoke extract; γ H2AX foci; vascular endothelial growth factor

Introduction

The larynx is an essential structure that coordinates swallowing, breathing, coughing, and phonation.¹ Due to its location at the intersection of the respiratory and digestive tracts, the larynx mucosal epithelium is susceptible to many immunologic challenges that are airborne and ingested. The hallmark pathology resulting from these challenges is laryngeal inflammation, which contributes to significant morbidity and costs. The cause of laryngitis, as identified by mucosal inflammation, is most often an inhaled irritant, gastric reflux, chronic infections, or overuse of the voice. The focus of this investigation is tobacco smoke due to the epidemiological studies linking this insult to diseases of the airway tract, including the larynx.²

The larynx houses the vocal folds, making the larynx the anatomical site of phonation. Dysfunctions of the vocal folds can have large societal costs, with estimations that nearly 10% of the US population have daily voice problems.³ This translates to a societal cost of approximately \$2.5 billion in the teaching profession alone. Reinke edema is another highly prevalent disease characterized by a swelling of the vocal fold lamina propria with symptoms of hoarseness.⁴ The dominant risk factor is tobacco smoking.⁵ Moreover, cigarette smoke is linked to nearly 30% of all cancer deaths⁶ and has been identified as a major risk factor for laryngeal squamous cell cancer.²

The immunological architecture of the larynx is poorly understood. The larynx marks the border between the immunoglobulin A (IgA) dominated upper airway and the IgG-dominated lower respiratory tract, and thus it is the site of many inhaled immunologic challenges.¹ Although there is a limited understanding of how mucosal inflammation is manifested in response to cigarette smoke, fibroblasts are known to play an important role in the paracrine regulation of the local microenvironment.⁷ Fibroblasts control the duration of the inflammatory infiltrate entering stromal tissues⁷ through the secretion of growth factors, chemokines, and cytokines.^{8–11} Moreover, isolated fibroblasts show tissue-specific characteristics related to gene expression and the markers of inflammation such as chemokine secretion.^{8,9,11} Therefore, cultured fibroblasts are a rational model for investigating the paracrine regulation of the human larynx in response to tobacco smoke in vitro.

Acute inflammation can mediate the host defense against microbial infection along with the tissue repair necessary for wound healing.^{10,12,13} Chronic inflammation, however, has been shown to have deleterious effects that can predispose tissues to many diseases, including cancer.^{10,12} The proposed molecular mechanisms of the pathogenesis of chronic inflammation in the lung associated with cigarette smoking are numerous. These include deregulated activation of the inflammatory transcriptional factor, nuclear factor κ B (NF- κ B), faulty integrin and tumor growth factor- β signaling leading to altered cell adhesion, changes in extracellular matrix and proteinase/antiproteinase balance, increased

angiogenesis, and genotoxic stress.¹⁴ Evidence points to a strong role for signal transduction leading to an increase of inflammatory cytokines associated with cigarette smoking.¹⁰ Genotoxic stress in the form of DNA double-strand breaks (DSBs) can elicit a nuclear signal that mediates the NF- κ B transcriptional activation of target genes, which include cytokines and chemokines.¹⁵ Recent studies in vitro have shown that cigarette smoke can induce the phosphorylation of the histone protein 2A variant H2AX (cH2AX),^{16,17} a developing clinical bio-marker for DSBs for different therapeutic treatment modalities.¹⁷ Therefore, the measurement of cytokines can be indicative of tissue-specific inflammation¹⁸ and possibly linked experimentally to certain genotoxic effects of cigarette smoke exposure.

The cholinergic anti-inflammatory pathway is a rapid physiological response to increases in tissue-specific cytokine levels under neural control. Studies show that the vagus nerve can upregulate acetylcholine levels in response to inflammation in certain tissues. Acetylcholine binds to the α 7-nicotinic acetylcholine receptor (α 7nAChR), which stimulates a reduction in cytokine secretion in cell types such as macrophages that express the receptor.¹⁹ Moreover, the α 7nAChR-regulated decrease of cytokines can be stimulated through the application of exogenous ligands, including nicotine.²⁰ Studies in a rat in vivo model have shown that this pathway could prove to be an effective therapeutic target in lung injury.²¹ However, the vagus innervated tissues of the larynx²² are not well characterized to be under the control of the cholinergic anti-inflammatory pathway. Here, we examined the expression of the α 7nAChR in human vocal fold fibroblasts (hVFFs), which are an anti-inflammatory target of nicotine, a major constituent of tobacco smoke. These results gave the context to ask whether cigarette smoke extract (CSE) can elicit a different inflammatory response compared to the endogenous proinflammatory cytokine, tumor necrosis factor- α (TNF- α).

Materials and Methods

Cell Culture

The isolation and growth of hVFFs have been published with the primary clone T21-hVFF²³ and immortalized clone A8-hVFF.²⁴ The immortalized bronchial epithelial cell line BEAS-2B was purchased from ATCC (Manassas, VA; CRL-9609). All cell types were used from passages 2 to 9 and cultured with Dulbecco modified Eagle medium (DMEM) with 10% fetal bovine serum (FBS), penicillin/streptomycin (100 μ g/mL), and $\times$ 1 nonessential amino acids. BEAS-2B (passage 2) undergoes squamous cell differentiation and forms higher-level structures in the presence of serum,²⁵ whereas hVFFs grow in a monolayer.^{23,24} The tissue culture supplies and all chemicals were purchased from Sigma-Aldrich (St Louis, MO). Human recombinant TNF α was purchased from EMD Millipore (Billerica, MA).

Cigarette Smoke Extracts

CSE protocol was adapted from previous studies.^{26,27} One full-strength Marlboro cigarette (Philip Morris USA, Richmond, VA) with the filter removed was combusted into a 30-mL syringe (Becton Dickinson Labware, Franklin Lakes, NJ) at a rate of three extractions per minute over 5 minutes. Smoke was bubbled into 10 mL of DMEM containing 0.5% FBS at room temperature (RT). Resulting CSE was adjusted to a pH of 7.4 and sterilized through a syringe with a 0.20- μ m pore size filter (Whatman GD/X sterile filter; GE Healthcare,

Piscataway, NJ). The optical density (OD) of the CSE was read by a spectrophotometer (SmartSpec Plus; Bio-Rad Laboratories, Hercules, CA) at 320 nm and diluted to 1.0 OD for consistency between experiments. Final CSE preparation was designated 10% and diluted to 1.0% and 0.5% for treatment of the cells. The diluted CSE was applied immediately to the cells.²⁷ Moreover, a plot of the OD versus CSE (10%, 5%, 3%, 2%, and 1%) was determined to be linear ($r^2 = 0.999$). Control was air bubbled into DMEM with 0.5% fetal calf serum (FCS) and processed similar to the 10% CSE.

Cell Viability Assay

A8-hVFFs at passage 9 were grown to near confluence in 12-well plates (Becton Dickinson Labware) and placed in DMEM containing 0.5% FCS for 24 hours to allow the cells to enter G₀ of the cell cycle.²⁸ Cells were exposed to CSE (0.5%, 1.0%, 3.0%) for 3 hours, washed with phosphate-buffered saline (PBS) twice, removed with trypsin, and isolated by centrifugation. Trypan blue (MP Biochemicals, Santa Ana, CA) exclusion was done to assess cell viability by using a hemocytometer under a light microscope.

Cytokine Analysis

A8-hVFFs at passage 9 were grown to near confluence ($\sim 5 \times 10^5$ /well) in 6-well plates and placed in DMEM containing 0.5% FCS for 24 hours. Cells were exposed to CSE (0.5%, 1.0%) and TNF α (10 ng/mL) for 3 hours. This concentration of TNF- α is used to induce an inflammatory response in cell culture.²⁹ Medium was removed, and the cells were washed twice with PBS. DMEM with 0.5% FCS (2 mL) was added for an additional 24 hours. Medium was isolated and transferred into Eppendorf tubes on ice followed by a brief high-speed centrifugation (4°C). The samples were quickly frozen on dry ice before storage at -80°C. There was no indication of cell death prior to isolation. The remaining cell monolayer was detached by trypsin and spun down in growth medium. Cell pellet was resuspended and placed on ice before trypan blue (0.04%) exclusion analysis at RT.³⁰ Cell counts using a hemocytometer under a light microscope (Olympus, Melville, NY) did not detect trypan blue-stained cells, indicative of cell death (data not shown). This is consistent with similar concentrations of CSE used to investigate the cytokine release in the absence of cell death.^{27,31} BioPlex Pro (Bio-Rad Laboratories) human cytokine assay was done with a multiplex plate measuring interleukin-1 β (IL-1 β), IL-1 receptor antagonist (IL-1ra), IL-6, IL-8, IL-10, IL-12, eotaxin, basic fibroblast growth factor (bFGF), monocyte chemotactic protein-1 (MCP-1), TNF- α , and vascular endothelial growth factor (VEGF) on a Bio-Plex 200 system. Protocol was done according to the manufacturer's instructions. The frozen medium was thawed, diluted, and immediately processed for analysis on the Bio-Plex 200 assay reader. Thawed samples were used once and discarded. Final concentrations were calculated from 27-Plex Group 1 standard curves analyzed by BioPlex Manager Software (Bio-Rad Laboratories). IL-1ra and IL-10 concentrations were below the standard curve and not reported. A second Bio-Plex assay was conducted with IL-6 and MCP-1 at higher dilutions. The approximate number of cells per milliliter of medium was estimated from the amount of medium applied to a confluent six-well plate (5×10^5 cells/2 mL = $\sim 250,000$ cells/mL).

Immunofluorescence Studies

$\alpha 7$ nAChR analysis—Cells (A8-hVFF, T21-hVFF, BEAS-2B) were seeded at 1×10^5 /well into 12-well plates (Becton Dickinson Labware) containing sterile glass cover slips (Fisher-brand, 15CIR-1D; Fisher Scientific, Pittsburg, PA) in normal growth medium. Cells were allowed to grow for 2 days, washed with PBS, and fixed with 4.0% formaldehyde (Ultrapure EM Grade; Polysciences, Warrington, PA) for 15 minutes. Cover slips were washed with PBS and blocked in 10% goat serum for 30 minutes at RT. A rabbit polyclonal antibody to $\alpha 7$ nAChR (H-302; Santa Cruz Biotechnology, Santa Cruz, CA)³² was added (1:50) to PBS/3% bovine serum albumin (BSA) and incubated overnight at 4 C. Coverslips were washed with PBS and incubated with fluorescein-coupled goat antirabbit secondary antibody (1:250; Invitrogen, Life Technologies, Grand Island, NY) in PBS/3% BSA with Hoechst 33342 (5 μ g/mL; Invitrogen, Life Technologies) for 1 hour at RT. Coverslips were rinsed with PBS and mounted (Vectashield; Vector Laboratories, Burlingame, CA) onto glass slides (Premium, Fisher Scientific). Slides were analyzed on a Nikon Eclipse E600 fluorescent microscope (Nikon Instruments, Melville, NY). Exposure time for the green (1/3 seconds) and blue excitation (1/700 seconds) was the same for each sample analyzed.

γ H2AX analysis—A8-hVFFs were seeded at 1×10^5 /well into 12-well plates containing sterile glass cover slips treated with fibronectin (50 μ g/mL). Cells were grown to near confluence and placed in DMEM containing 0.5% FCS for 24 hours before exposure to CSE for 3 hours. Cells were rinsed with PBS, fixed with 4.0% formaldehyde for 15 minutes, and then washed with 150 mM Tris-buffered saline (TBS)/0.1% Triton X-100. TBS/0.5% Triton X-100 was placed onto the coverslips for 5 minutes and removed before the addition of TBS/10% goat serum/0.5% gelatin for 30 minutes. The anti-phospho-Histone H2A.X (Ser139), clone JBW301 antibody (EMD Millipore) was used at a dilution of 1:500 in TBS/3% BSA for 1 hour at RT. Coverslips were washed with TBS/0.1% Triton X-100 before incubation with a fluorescein-attached goat antimouse secondary antibody (Invitrogen) at a dilution of 1:250 in TBS/3% BSA/Hoechst 33342 for 1 hour at RT. Coverslips were washed and processed for immunofluorescence at a magnification of 40 $\times$. Exposure time for the green and blue excitation was the same for each sample analyzed. The analysis of γ H2AX nuclear foci associated with DNA double-strand breaks¹⁷ was with Foci-Counter software.³³

Polymerase Chain Reaction

Total RNA was isolated from cells grown to 50% confluence by using a RNeasy Mini Kit (Qiagen, Valencia, CA). Generation of cDNA was done with an Omniscript Reverse Transcriptase Kit (Qiagen). Manufacturer's instructions were used for both assays. Random hexamers (10 μ M; Integrated DNA Technologies, Coralville, IA) and 150 ng/mL of RNA as the template was used to generate cDNA. A nested polymerase chain reaction (PCR) method was used to increase the amplification and specificity of the $\alpha 7$ nAChR transcript,^{34,35} with homo sapiens ribosomal protein S14 (RPS14) used as the control (Table 1).³⁶ All primer sets (Integrated DNA Technologies) span introns and will not amplify genomic DNA at the indicated molecular weights. The primer set used to generate the 375 base pair (bp) product of the $\alpha 7$ nAChR has been characterized (Table 1).³⁴ PCR reactions were run on a Peltier Thermal Cycler (MJ Research, Waltham, MA). Nested PCR was initiated with the 375-bp

product primer set (0.5 μ M each) in a volume of 50 μ L with 5 μ L of the template cDNA, $\times$ 1 buffer, 2.0 mM MgCl₂, 0.2 mM dNTPs, and Go Taq Hot Start DNA Polymerase (Promega, Madison, WI). PCR was started with a denaturation at 95°C for 2 minutes, followed by 35 cycles of 95°C for 30 seconds, 60°C for 15 seconds, and 72°C for 1 minute. Final extension was at 72°C for 10 minutes. The 319-bp product primer set (0.5 μ M each; Table 1) was run with 5 μ L of the completed PCR reaction under the same conditions. RPS14 was run during the first cycle set under the same conditions. Primer products along with a PCR DNA ladder (Promega) were run on a 2.0% agarose gel stained with ethidium bromide. The resulting gel picture was taken by a Gel Documentation System (UVP, Upland, CA).

Statistics

One-way repeated measures analysis of variance (ANOVA) determined whether there was a difference in the mean values of cytokine, chemokine, and growth factor levels with control, CSE, and TNF- α . Results shown are from three independent experiments. A post hoc Fisher least significant difference (LSD) test was used to determine multiple paired comparisons to the control. One-way ANOVA ($n = 1$) followed by Fisher LSD test examined the number of γ H2AX nuclear foci and cell viability. A probability value $<.05$ was considered significant.

Results

Studies examined the mRNA expression of the α 7nAChR in hVFFs by using the specificity of nested PCR analysis (Fig. 1, Table 1).^{34,35} Results showed that A8-hVFFs and T21-hVFFs express α 7nAChR mRNA levels similar to the BEAS-2B cells known to express the receptor (Fig. 1A).³⁷ RPS14³⁶ used as a control demonstrated that the concentration of cDNA was consistent in each sample. Further studies showed that transcript levels of the α 7nAChR decreased with later passage hVFFs (Fig. 1B). Immunofluorescence analysis of nonpermeabilized cells illustrated that the protein expression of the α 7nAChR decreased with the passage number in A8-hVFFs (Fig. 2). However, there was not a similar decrease in the protein levels in the later passage T21 cells.

Later passage A8-hVFFs (passage 9) were exposed to varying concentrations of CSE (0.5%, 1.0%, 3.0%) to assess the induction of DSBs with γ H2AX analysis.^{16,17}

Immunofluorescence studies showed that 3% CSE exposure for 3 hours elicited a significant increase in the number of γ H2AX nuclear foci compared to control, and 0.5% and 1.0% CSE (Fig. 3A, B). There was no change in cell viability with CSE exposure as measured by trypan blue exclusion at this time point (Fig. 3C).

To determine whether cigarette smoke could elicit an inflammatory cytokine response in comparison to TNF- α , later passage A8-hVFFs (passage 9) were exposed to CSE. Results from the multiplex cytokine analysis showed that TNF- α significantly increased soluble levels of IL-1- β , IL-6, IL-8, eotaxin, bFGF, MCP-1, and TNF- α at 24 hours post-treatment (Fig. 4, Table II). IL-12 and VEGF did not change with TNF- α treatment, and CSE (0.5%) did not elicit a response in the A8-hVFFs. However, CSE (1.0 %) significantly increased soluble levels of VEGF (Fig. 4A, Table II), which was relatively specific in comparison to the other cytokines, chemokines, and growth factor tested. These results showed that CSE (1.0%) and TNF- α produce a markedly different inflammatory response.

Discussion

Our studies showed that hVFFs express the $\alpha 7$ nAChR, a necessary component of the cholinergic anti-inflammatory pathway. The vocal folds are part of the larynx region innervated by the superior and recurrent laryngeal nerves of the vagus nerve system,²² known to regulate the cholinergic anti-inflammatory pathway in other tissues.²⁰ Studies here showed that hVFFs express the $\alpha 7$ nAChR through mRNA and immunofluorescence analysis consistent with studies by others.^{32,34,35,38} Later passage T21-hVFFs expressed a reduction in mRNA levels and did not correlate with a decrease in protein levels. These results are consistent with studies by others showing that mRNA levels of $\alpha 7$ nAChR can decrease in cultured cells over time without a concomitant reduction in protein levels.³⁹ Moreover, post-translational processing of $\alpha 7$ nAChR is an important step in the expression of this receptor.^{40–42} This additional regulatory step further suggests why a reduction in mRNA levels is not always consistent with a concomitant decrease in protein levels in nonpermeabilized cells.⁴⁰ We chose to examine the later passage A8-hVFFs due to the lower protein levels of $\alpha 7$ nAChR because of the possibility that the significant levels of nicotine contained in CSE^{43,44} could decrease the inflammatory response. Nicotine is an effective activator of $\alpha 7$ nAChR, which can inhibit the inflammatory effects of TNF- α and endotoxin.⁴⁵ Epidemiological and clinical studies have shown that increases of serum concentrations of nicotine from cigarette smoking correlate with the remission of inflammatory bowel disease.⁴⁶ Moreover, this effect of cigarette smoking could be mediated through the reduction of inflammatory cytokine levels in the colonic mucosa of subjects with this disease.⁴⁷ Therefore, because pharmacological activation of $\alpha 7$ nAChR is under development,¹⁹ we have initiated the examination of hVFFs as a cell type to inhibit the paracrine effects of cigarette smoke exposure.

The present in vitro study used a methodology known to extract the water-soluble components of cigarette smoke⁴⁸ to expose lamina propria fibroblasts. Cigarette smoke is a complex mixture that can be divided into gas and tar phases,⁴⁹ which collectively contains more than 4,000 chemicals.¹⁴ Many of the chemicals found in the tar or particulate phase are water soluble and released into the biological fluids covering the airway tract.⁴⁹ Similarly, a layer of fluid covers the vocal fold, establishing a hydrated barrier to insults,⁵⁰ mainly due to the intercellular tight junctions important for maintaining the structure of the airway epithelium.⁵¹ However, a physiological model of airway surface fluid at the basal state suggests that water can pass through tight junctions into interstitial tissue.⁵² Moreover, the movement of fluid through tight junctions has been shown with vocal fold epithelium treated with the inflammatory insult histamine.⁵³ This indicates there is a plausible route of exposure from deposited cigarette tar onto the vocal fold epithelium through a paracellular route to the underlying vocal fold lamina propria. Here, in agreement with previous studies of cigarette smoke exposure of human pulmonary adenocarcinoma cells,¹⁶ we showed that 3.0% CSE induced γ H2AX nuclear foci in growth-inhibited hVFFs. These results are within the range of the γ H2AX nuclear foci formed in postmitotic neurons treated with the chemotherapeutic agent camp-tothecin.⁵⁴ However, our studies did not detect significant levels of γ H2AX with 1% CSE exposure compared to controls. Generally, γ H2AX is rapidly detected¹⁷ within the time frame of exposure used in our studies, including with cigarette

smoke.¹⁶ Therefore, our results suggest that the induction of VEGF levels with 1.0% CSE is independent of the DSBs associated with carcinogenesis.¹⁷

TNF- α significantly induced a greater number of paracrine factors analyzed in our studies compared to CSE treatment. This potent cytokine mediates a proinflammatory response through the activation of the transcriptional factor NF- κ B in many different types of cells.⁵⁵ NF- κ B expressed in the cytoplasm can be activated by inflammatory stimuli, which initiates the trans-location of the transcription factor to the nucleus. DNA binding allows NF- κ B to coordinate the transcriptional regulation of genes that control acute and adaptive immunity, as well as inflammation.^{14,56,57} However, studies show that fibroblasts have constitutive activation or nuclear expression of the NF- κ B family member RelB.^{58,59} RelB constitutive activation in fibroblasts reduces the response of certain chemokines and cytokines to inflammatory stimuli mediated by NF- κ B.^{58,59} We have observed RelB expression in the nucleus of A8-hVFFs by immunohistochemistry consistent with constitutive activation (data not shown).⁶⁰ Here, our studies showed that TNF- α treatment elicited a significant but not marked induction with IL-1 β and TNF- α . In agreement, RelB-deficient fibroblasts induced a greater increase in IL-1 β and TNF- α mRNA with lipopolysaccharide activation of NF- κ B compared to normal fibroblasts.⁵⁹ Moreover, intranasal injections of an adenovirus expressing RelB showed a reduction in the inflammatory response to cigarette smoke exposure in the murine lung. CSE is a known activator of NF- κ B.^{61–63} Our results did not show significant changes in TNF- α ,¹¹ eotaxin,⁶⁴ or IL-8,⁶¹ which are induced with CSE in other cell types.^{11,61,64} Microarray analysis of genes differentially expressed in subjects with Reinke edema showed an increase in the expression of RelB compared to subjects with vocal fold polyps, a benign tumor.⁶⁵ The gene induction of RelB in Reinke edema tissue correlated with a reduced expression of inflammation-related genes compared to vocal fold polyps.^{58,59} Collectively, our studies corroborate with clinical and in vitro studies suggesting that fibroblasts are a plausible therapeutic target in chronic inflammation⁷ due in part to constitutive expression of RelB.⁶⁶

Our results in vitro may give insight into the potential role of hVFFs in the paracrine regulation of pathological blood vessel formation in Reinke edema.^{67,68} CSE elicited an increase in VEGF, which is an important growth factor regulating angiogenesis and vascular permeability.⁶⁹ Clinical studies using immunohistochemistry have shown that cells in the interstitial tissue of the vocal fold express increased VEGF protein levels in Reinke edema compared to normal subjects.⁶⁷ Although fibroblasts are the main resident of the vocal fold,⁷⁰ the specific cell type expressing VEGF was not delineated in these studies. Moreover, the vasculature was leaky with dilated, looping, and branching blood vessels proposed to cause edema and tissue swelling.^{67,68} This type of pathogenic vasculature is observed in diseases such as cancer and age-related macular degeneration.⁷¹ Preclinical studies show that anti-VEGF therapy can inhibit the growth of new blood vessel formation while allowing the normalization of the pathological blood vessels in some disease models. Certain anti-VEGF drugs are approved for the treatment of diseases such as cancer and glaucoma.⁷¹ Thus, our studies along with others⁶⁷ suggest that fibroblasts of the vocal fold lamina propria may be a cellular target for anti-VEGF therapy to prevent or treat Reinke edema.⁶⁷

Reinke edema mainly associated with cigarette smoking can be difficult to diagnose because of the histo-logical similarities with other benign lesions of the vocal fold lamina propria such as polyps and nodules.⁷² Nicotine is the psychoactive compound in tobacco that causes the strong addiction to cigarettes,⁷³ making long-term treatment of Reinke edema problematic.^{74,75} Studies have shown that smoking cessation alone does not induce the complete regression of Reinke edema,⁷⁵ and there is a significant incidence of recurrence following surgical treatment.⁷⁴ Moreover, the scarring from surgery can change the viscoelastic properties of the lamina propria, causing dysphonia.^{76,77} The present study showed that CSE exposure of hVFFs elicited an increase in the level of soluble VEGF, which potentially could be exploited clinically to facilitate the diagnosis of Reinke edema. Fine-needle aspiration of the vocal fold lamina propria has been successfully tested for the analysis of cells and fluid in an animal model.^{78,79} There were no changes in the measured viscoelastic properties of the vocal fold with this minimally invasive procedure.⁷⁸ A similar method has been used to extract the aqueous humor fluid from the eye⁸⁰ to analyze the soluble levels of VEGF in certain forms of glaucoma⁸⁰ and macular edema.⁸¹ Therefore, early detection of soluble levels of VEGF with fine-needle aspiration may allow for the diagnosis of early stage Reinke edema, suggesting that preventative therapies are necessary before the onset of dysphonia and surgical treatment.

Conclusion

An unbiased high content analysis of inflammatory cytokines, chemokines, and growth factors showed that hVFFs elicited a relatively specific proangiogenic response as measured by increasing levels of soluble VEGF with CSE exposure. Because VEGF has been shown to be overexpressed in lamina propria cells with Reinke edema, we suggest that the measurement of soluble VEGF levels by fine-needle aspiration could allow for early diagnosis before the onset of dysphonia and surgery. Moreover, hVFFs express the $\alpha 7$ nAChR, which is a developing pharmacological target to reduce pathogenic inflammation through activation of the cholinergic anti-inflammatory pathway.

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Fig. 1.

Nested polymerase chain reaction (PCR) showed that human vocal fold fibroblasts (hVFFs) express the $\alpha 7$ -nicotinic acetylcholine receptor ($\alpha 7$ nAChR) mRNA transcript. Nested PCR with $\alpha 7$ nAChR was done according to Materials and Methods. (A) hVFFs expressed mRNA levels similar to the positive control BEAS-2B. Agarose gel of the upper panel shows the PCR product of the $\alpha 7$ nAChR in lanes 1 to 3 indicated by 300 bp. Lower panel depicts the RPS14 PCR product as indicated by 150 bp. (B) PCR product of the $\alpha 7$ nAChR is not detected in higher passage hVFFs. The upper panel depicts the $\alpha 7$ nAChR band (lane 1, lane 2) in lower passage hVFFs compared to the absence of the product in lanes 3 and 4 with higher passage hVFFs. RPS14 shows consistent concentrations of cDNA were used from each sample.

Fig. 2.

Early and later passage A8–human vocal fold fibroblasts (hVFFs) showed a reduction in the expression of the $\alpha 7$ -nicotinic acetylcholine receptor ($\alpha 7$ nAChR) with immunohistochemistry. The first column shows the expression of the $\alpha 7$ nAChR in nonpermeabilized cells as detected by immunofluorescence. DNA staining by Hoechst 33342 is shown in the second column. The third column depicts the first two rows superimposed, showing the expression of the $\alpha 7$ nAChR in contrast to the nucleus. The cell type and passage number are designated on the side of the figure. BEAS-2B cells are the positive control. These cells undergo squamous cell differentiation in serum and form higher level structures in cell culture. BEAS-2B cells at the bottom of the panel are out of the microscope phase, with the middle portion of the nest of cells expressing the $\alpha 7$ nAChR. Results are from one experiment done in triplicate. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

Fig. 3.

Cigarette smoke extract (CSE; 3%) exposure induced γ H2AX nuclear foci in A8–human vocal fold fibroblasts. (A) Later passage cells were exposed to CSE at varying concentrations for 3 hours and analyzed by immunofluorescence at 40 $\times$. The represented result from γ H2AX fluorescence was superimposed onto DNA staining with the indicated treatment. (B) Graphical depiction of the number of nuclear foci as analyzed by FociCounter software. Results are the average of three determinants from one experiment. There were approximately 150 nuclei analyzed for each treatment. *Significant difference ($P < .05$) between the mean values of the treatment group. #Significant difference ($P < .05$) in the mean value compared to the control with treatment. (C) Graphical depiction of the number of viable cells with CSE exposure. Cells were treated as in A and examined for viability by trypan blue exclusion at 3 hours. There were approximately 600 cells counted for each treatment. The experimental design and statistical analysis are as in B. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

Fig. 4.

Multiplex assay of cytokines, chemokines, and growth factors was used to analyze the response of A8–human vocal fold fibroblasts to cigarette smoke extract (CSE) exposure. Later passage cells were exposed for 3 hours with CSE (0.5%, 1.0%), tumor necrosis factor- α (TNF- α ; 10 ng/mL) along with control. Cells were washed with phosphate-buffered saline, and low serum medium was added for an additional 24 hours. The medium was isolated according to Materials and Methods. Each bar represents the mean $\pm$ standard deviation from three independent experiments. *Significant difference ($P < .05$) between the mean values of the treatment group. #Significant difference ($P < .05$) in the mean value compared to the control with treatment. (A) CSE does affect interleukin (IL)–1 β , IL-12, eotaxin, basic fibroblast growth factor (bFGF), and TNF- α levels. Graph with IL-1 β , IL-12, eotaxin, bFGF, and TNF- α ranges from 0 to 400 pg/mL. (B) CSE (1.0%) increases vascular endothelial growth factor (VEGF) levels. The break in the y-axis of the IL-8 and VEGF graph ranges from 6,500 pg/mL to 10,000 pg/mL to delineate the concentrations of monocyte chemoattractant protein-1 (MCP-1) and VEGF.

Table 1

Primers Used for the Nested Polymerase Chain Reaction and Control.

Gene	GeneBank No.	Cycle Set	Forward Primer, 5'-3'	Reverse Primer, 5'-3'	Product, bp
<i>α7nAChR</i>	NM_000746.1	1	GGAGCTGGTCAAGAACTACA	CAGCGTACATCGATGTAGCA	375
		2	TCCCTTGGAGAGGGCCCGTGG	GCCTGGAGGCAGGTACTGGCA	319
<i>RPS14</i>	NM_002025070.1	1	TCAACGGCCCTACACATCAAACT	CTGCGAGTGTGTCTCAGAGG	157

Table II

VEGF Levels Are Induced at 24 Hours After Exposure With Cigarette Smoke Extract (1.0%).

Treatment	IL-1 β	IL-12	Eotaxin	bFGF	TNF- α	IL-6	IL-8	MCP-1	VEGF
Control	11 $\pm$ 1 [*]	40 $\pm$ 4	328 $\pm$ 27 [*]	113 $\pm$ 8 [*]	103 $\pm$ 11 [*]	14,898 $\pm$ 1,251 [*]	19,266 $\pm$ 475 [*]	3,803 $\pm$ 249 [*]	1,609 $\pm$ 321 [*]
0.5%	9 $\pm$ 1	41 $\pm$ 6	292 $\pm$ 17	100 $\pm$ 6	89 $\pm$ 6	15,989 $\pm$ 2,968	16,009 $\pm$ 3,131	4,439 $\pm$ 330	1,619 $\pm$ 459
1.0%	11 $\pm$ 1	41 $\pm$ 6	332 $\pm$ 15	119 $\pm$ 3	110 $\pm$ 11	15,448 $\pm$ 2,442	28,729 $\pm$ 6,460	4,4321 $\pm$ 624	2,968 $\pm$ 413 [†]
TNF- α	15 $\pm$ 1 [†]	38 $\pm$ 5	420 $\pm$ 38 [†]	137 $\pm$ 12 [†]	147 $\pm$ 10 [†]	26,044 $\pm$ 6,086 [†]	47,139 $\pm$ 14,703 [†]	5,512 $\pm$ 869 [†]	1,419 $\pm$ 175

Results are expressed as pg/mL. Data represent the mean $\pm$ standard deviation from three individual experiments.

^{*} Significant difference ($P < .05$) between the means of the treatment group.

[†] Significant difference in the mean value compared to the control.

bFGF = basic fibroblast growth factor; IL = interleukin; MCP-1 = monocyte chemoattractant protein-1; TNF- α = tumor necrosis factor- α ; VEGF = vascular endothelial growth factor.