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QUANTIFICATION OF INFLAMMATORY MARKERS IN LARYNGOTRACHEAL STENOSIS

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Abstract

Objectives—1) Develop a novel method for serial assessment of gene and protein expression in laryngotracheal stenosis (LTS) 2) Assess cytokine expression and determine an immunophenotype in LTS.

Study Design—A matched comparison of endolaryngeal brush-biopsy samples from laryngotracheal scar and normal airway.

Setting—Tertiary care hospital, 2015–2016

Methods—Brush-biopsy specimens of laryngotracheal scar and normal trachea were obtained from seventeen LTS patients at the time of OR dilation and were used for protein and RNA extraction. Gene expression of the T_H1 cytokine Interferon- γ (INF- γ), T_H2 cytokine Interleukin (IL) – 4, Transforming Growth Factor – β , and Collagen-1 (Coll1) was quantified using quantitative RT-PCR. Cytokine analysis was performed with flow cytometry using a cytometric bead array.

Results—LTS specimens demonstrated a 13.68 fold increase in Coll1 gene expression compared to normal ($p < 0.001$, $n = 17$). Additionally, IL-4 gene expression showed a 3.76-fold increase ($p < 0.001$, $n = 17$) in LTS scar. When stratified into iatrogenic LTS (iLTS) and idiopathic subglottic stenosis (iSGS) cohorts INF- γ gene expression was significantly increased in iSGS ($p = 0.011$). Soluble cytokine measurements were below the limit of detection for reliable quantification and thus could not be assessed.

Conclusions—Brush biopsies from LTS samples can be successfully utilized for RNA extraction and demonstrate the expected increase in Coll1 gene expression associated with LTS. Preliminary gene expression suggests abnormal collagen production may be mediated by the T_H2

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cytokine IL-4, and that increased INF- γ expression may represent a key difference between iLTS and iSGS. Further analysis of soluble cytokines is needed to confirm these findings.

Introduction

Laryngotracheal stenosis (LTS) is the pathologic narrowing of the larynx, subglottis, and/or trachea. This narrowing is secondary to excessive scar tissue formation and often leads to dysphonia and/or dyspnea in patients. LTS can result from multiple etiologies including post-intubation injury (iatrogenic), autoimmune, radiation-induced, or idiopathic, with most cases of LTS being iatrogenic and idiopathic in nature. Distinct clinical profiles exist between iatrogenic LTS (iLTS) and idiopathic subglottic stenosis (iSGS). iSGS primarily affects middle age Caucasian females with no underlying autoimmunity or history of previous intubation, while iLTS affects a more heterogeneous population.^{1,2} Despite recognition of these unique demographics, our understanding of the pathogenesis of LTS and how it varies between each etiology is poorly understood and is a barrier to improving medical therapies for LTS.

Research investigating the pathogenesis of LTS is sparse secondary to the rarity and heterogeneity of the disease. For iLTS, current literature suggests that an initial epithelial and subepithelial injury leads to a prolonged and dysregulated wound healing response that promotes the deposition of pathologic scar in the airway.^{3,4} Efforts to characterize the aberrant wound healing in iLTS have utilized in-vivo animal models which demonstrate a critical role for the adaptive immune response in the development of airway stenosis thus implicating an immune mechanism in the pathogenesis of iLTS.^{5,6} Underlying mechanisms for the pathogenesis of iSGS are less well defined. Several hypotheses for the pathogenesis of iSGS exist and include changes in estrogen and estrogen-level receptors and microtrauma to the airway; however, these hypothesis have not been clearly demonstrated.⁷ More recently, characterization of the local microbiota and immunological milieu in iSGS has demonstrated unique populations of bacteria and has identified changes in the IL-17 signaling axis thus implicating a potential immunological mechanism for iSGS as well.^{7,8}

Given the proposed role of the inflammatory system in the pathogenesis of both iLTS and iSGS the development of a method for minimally invasive assessment of inflammation in human LTS would be beneficial in improving our understanding the immunopathogenesis of this disease. Additionally, characterization of serial changes in inflammation in both iLTS and iSGS would provide information about disease progression and could potentially identify a biopredictive marker that would allow for risk stratification and help the clinician and patient guide treatment plans. Ideally, this method would be applicable to an office-based setting so that serial assessment of inflammation after OR-based intervention could be monitored. Additionally, the platform for this test would be a single modality that provides information at the genetic and post-translational level. The goal of this study was to assess the efficacy of novel brush biopsy technique for measurement of inflammatory cytokines and determination of an immunophenotype in iLTS and iSGS patients.

Methods

Patient Selection

Brush-biopsies of human laryngotracheal scar and normal appearing areas of the airway were procured during routine dilation of subglottic and tracheal stenosis in seventeen consecutive patients with a diagnosis of iSGS or iLTS between 2015 and 2016. All patients in this study signed informed consent and approval for this study was granted by the Johns Hopkins Institutional Review Board (NA_00078310). Clinical history was abstracted and reviewed for each patient.

LTS Tissue Sampling

Brush-biopsies of human laryngotracheal scar and normal appearing areas of the airway were obtained using a 1.0mm diameter bronchial cytology brush (ConMed, Utica, NY). Specimens were procured at the time of routine operating room dilation of subglottic and tracheal stenosis. Brushes biopsy samples were immediately housed in 1.5cc sterile conical tubes and placed on dry ice until they could be stored at -80°C . Brush biopsy samples were stored for no longer than 4 weeks prior to processing.

Gene Expression Analysis by Quantitative Real-time Polymerase Chain Reaction

RNA was extracted and purified from brush-biopsies using a RNeasy Microkit as part of the AllPrep RNA/Protein kit (Qiagen, Valencia, California). RNA was quantified using a nanodrop 2000 spectrophotometer (Thermo Scientific, Waltham, Massachusetts) and was then used for the creation of complimentary DNA (cDNA). Gene expression was assessed by quantitative real-time polymerase chain reaction (qRT-PCR) using a StepOnePlus Real Time PCR system (Life Technologies, Carlsbad CA) and Power SYBR Green Mastermix (Life Technologies). cDNA samples were diluted 1:30 and 1uL was combined with 10uL of Mastermix, 8uL of ddH₂O, and 0.5uL of 10 uM forward and reverse primer for the gene of interest (total volume of 20uL). Each reaction well underwent 40 cycles of a denaturation step at 95°C for 15 seconds, followed by annealing and extension at 60°C for 60 seconds. The cycle threshold for gene product detection (CT) of the gene of interest was normalized against the CT value for the reference gene β -Actin (CT) for all samples and then compared to normal controls (CT). Gene expression is presented as the relative fold change calculated by $2^{-\text{CT}}$. All samples were investigated in triplicate. Gene expression was quantified for the fibrosis-related gene Collagen-1 (Coll1), as well as the cytokines Transforming Growth Factor- β (TGF- β), Interleukin (IL)-4, and Interferon- γ (INF- γ) (Table 1).

Flow Cytometry of Inflammatory Cytokines

Protein was extracted from brush-biopsy samples using RIPA buffer (50mM Tris-HCL, 150mM NaCl, 2% NP-40, 1% Na Deoxycholate, 0.2%SDS), 10 μM PMSF, 50 μM EGTA, 10 μM Na Orthovanadate, 0.5 mM Na Fluoride, 1% cOmplete Protease Inhibitor Cocktail (Sigma-Aldrich, St. Louis, MO, USA). Protein concentrations were determined using a standard curve assay. A panel of six cytokines including IL-2, 4, 6, 10, Tumor Necrosis Factor (TNF), and (INF- γ) were detected using a commercially available cytometric bead

array (BD Biosciences, San Jose, CA, USA) and analyzed using a Accuri C6 Plus Flow Cytometer (BD Biosciences, San Jose, CA, USA). Data was normalized and quantified on a standard curve.

Statistical Analysis

Differences between LTS scar and normal trachea samples were compared using a non-parametric Wilcoxon matched-pairs signed-rank test. A Mann-Whitney test was used to assess differences between the iSGS and iLTS subgroups. Results are displayed as the mean fold change and 95% confidence interval (CI). A type I error rate (α) of less than 0.05 was considered statistically significant. A bonferroni correction (α/m) was applied to adjust for multiple hypotheses ($m=3$) making the level of statistical significance in this study at a p-value less than 0.0167. Data analysis was performed using Prism software (GraphPad Software Inc., La Jolla, CA, USA).

RESULTS

Demographics

Brush-biopsy specimens were collected from a total of 17 patients with a diagnosis of iLTS (n=9) or iSGS (n=8). The average age of this group was 43.8 years. The majority of patients in this group were Caucasian (82.1%) and female (94%) (Table 2). There were no significant differences in age, sex, or race between the iatrogenic and idiopathic cohort of patients. There was an increased propensity of patients who were tracheostomy dependent in the iatrogenic cohort (55 vs 0%, $p=0.03$)

Collagen-1 Gene expression is increased in areas of LTS scar

Endolaryngeal brush biopsy was successfully and safely obtained in all 17 patients undergoing OR dilation without complication. Sufficient RNA quantity and quality was obtained for gene expression analysis in all samples. Quantification of RNA ranged from 10–100 ug/uL per sample. Analysis of brush-biopsy samples for coll1 gene expression revealed a 13.68 fold (n=17, $p<0.001$, 95% CI: 4.29–43.65) increase in LTS scar compared to normal trachea (Figure 1a). When stratified into subgroups there was no significant difference in coll1 gene expression between the iSGS and iLTS subgroups. However, while both groups demonstrated increased coll1 gene expression in LTS scar compared to matched controls, statistical significance was only demonstrated in the iSGS subgroup (n=8, $p=0.001$, 95% CI: 6.72–157.91) (Figure 2a).

Interleukin-4 expression is increased in areas of LTS scar

Gene expression analysis of CD4⁺ T-cell related cytokines revealed an upregulation of key cytokines. In this series of patients, the T_H2 related cytokine IL-4 had a 3.76 fold increase in gene expression (n=17, $p<0.001$, 95% CI: 1.97–7.38) when looking at the entire population (Figure 1b). When stratified into iSGS and iLTS subgroups IL-4 expression in each group remained significantly elevated compared to matched normal controls. There was no difference in IL-4 expression between the iSGS (Fold Change = 4.61, n=8, 95% CI: 1.51–14.02) and iLTS groups (Fold Change = 3.12, n=9, 95% CI: 1.22–7.96) (Figure 1b) reported as average fold change compared to matched normal controls.

Interferon- γ expression is increased in areas of Idiopathic LTS scar

Analysis of the T_H1 related cytokine INF- γ revealed a non-significant increase in LTS specimens compared to matched normal controls (Fold Change: 1.57, n=17, 95% CI: 1.04–2.58, p=0.048) (Figure 1c). However, when stratified into idiopathic and iatrogenic cohorts the iSGS group demonstrated a significant elevation in INF- γ gene expression compared to matched normal controls (Fold change = 2.83, n=8, 95% CI: 1.60–5.00,). Furthermore, INF- γ expression in the iSGS samples was significantly higher than iLTS samples (Fold change = 0.948, n=9, 95% CI: 0.56–1.82 p=0.011) (Figure 2c). There was no significant difference in INF- γ expression in iLTS samples compared to matched normal controls (p=0.82). Gene expression analysis of the profibrotic cytokine TGF β did not result in a significant differences between groups as well as matched normal controls (Figure 1d/2d).

Protein Quantification and Flow Cytometric Assessment of Cytokines

Using the technique described protein was extracted and purified from brush biopsy samples. Protein concentrations ranged from 3.87–43.89 mg/mL per sample. However, measurement of the T_H1 and T_H2 related cytokines IL-2, 4, 6, 10, Tumor Necrosis Factor- α , and INF- γ using flow cytometry revealed that cytokines in the protein extracted from the brush biopsy were below detectable levels and thus not quantifiable.

Discussion

In this study, we demonstrate a novel method for endolaryngeal sampling of the epithelial and sub-epithelial immune microenvironment in LTS. Using this method the changes in gene expression of immunological markers in LTS tissue can be quantified in a minimally invasive fashion. Given that LTS is chronic fibroinflammatory disorder, characterization of the molecular and immunological changes that potentiate this disease process is vital to improved understanding of its pathogenesis and has the potential to improve treatment strategies for LTS. Additionally, correlation of changes in key markers of inflammations in LTS to disease status could lead to the identification of a novel biopredictive marker capable of influencing treatment decisions.

The endolaryngeal brush biopsy technique described in this study was able to consistently obtain adequate genetic material for PCR analysis. Using this technique all samples from LTS scar and normal appearing trachea had adequate RNA for the creation of complimentary DNA for PCR analysis. Given that LTS is a disease hallmarked by increased collagen deposition, the robust increase in coll1 gene expression in the LTS samples compared to normal controls serves as validation that our method is accurately capturing diseased and normal trachea. Additionally, when the overall cohort was stratified into idiopathic and iatrogenic subgroups, albeit non-significant, the iatrogenic subgroup had lower levels of coll1 gene expression compared to the idiopathic group and did not meet significance when compared to matched normal controls. These trends may reflect that iatrogenic LTS is a less active process at the time of operative dilation while iSGS continues to have ongoing inflammation and fibroblast activation. However, further investigation is needed to confirm these findings.

When analyzing the gene expression of T-cell related cytokines of interest, this studies cohort revealed a significant increase in IL-4 expression in LTS scar compared to normal controls (figure 1b) and these differences were maintained in both subgroups upon stratification (figure 2b). Given that IL-4 is a secreted cytokine with a wide range of function including immunosuppression, T_H2 T-cell and M2 macrophage differentiation, and chronic fibrosis this result was an anticipated finding.^{9,10} IL-4's association with multiple fibroproliferative diseases, including idiopathic pulmonary fibrosis, cardiac fibrosis, and systemic sclerosis has prompted investigations to identify its role in fibrosis.^{11–13} In idiopathic pulmonary fibrosis disease progression is associated with persistent IL-4 elevation.¹⁴ Additionally, in-vitro models have demonstrated IL-4's ability to regulate fibroblast function including promoting myofibroblast differentiation, collagen deposition, and proliferation.¹⁵ However, in-vivo models investigating the effects of IL-4 in fibrosis have not been transparent. IL-4 has a demonstrated critical anti-inflammatory role that promotes survival after initial injury in bleomycin-induced models of IPF but conversely potentiates the long-term deposition collagen that hallmarks disease progression.¹⁰ While the exact role of IL-4 in fibrosis remains to be fully elucidated, its increase expression in LTS tissue in this study indicates that a similar immunological mechanism between LTS and other fibroproliferative diseases may exist but further investigation is needed.

Recent evidence investigating different etiologies of LTS suggests that there are key differences in the pathogenesis of iSGS and iLTS. In iSGS the IL-23/IL-17A signaling axis has been demonstrated to be upregulated compared to iLTS and suggests that there are differences in the pathogenesis of this disease as it relates to the immune response.¹⁶ The differences in INF- γ expression between the two subgroups of LTS in this study further supports that different underlying immunological mechanisms in iSGS and iLTS exist. In the current study, when samples were stratified into idiopathic and iatrogenic subgroups gene expression of INF- γ was significantly elevated in the iSGS group. While the source this increased INF- γ expression cannot be determined, gamma-delta T-cells which have been shown to be increased in iSGS are potent producers of INF- γ and may explain this observed increase.^{16,17} Another potential explanation for the increase in INF- γ expression in iSGS is the propensity for bacterial colonization by mycobacterium species in iSGS.⁸ Previous studies have demonstrated that Mycobacteriums antigenicity invokes a robust release of INF- γ from T-cells, natural killer cells, and macrophages.^{18,19} Like IL-4, the role of this increased INF- γ expression in iSGS is also unclear. Interestingly, INF- γ has previously been thought to be an anti-fibrotic cytokine that is deficient in many fibroproliferative diseases and thus increasing INF- γ has been a strategy behind many interventions aimed at reducing fibrosis.^{20–23} This seemingly contradictory finding in iSGS necessitates further studies to define INF- γ 's role in iSGS.

The use of the novel brush biopsy technique introduced in this study offers a facile approach for immunological sampling in LTS that minimizes the trauma associated with conventional biopsies and has potential for office-based application through the working channel port of a bronchoscope. Currently, molecular and immunological assessment of LTS is restricted by the ability to only obtain samples at the time of OR-based intervention, a time point that likely does not capture the key molecular and inflammatory changes that potentiate restenosis in the weeks following intervention. Given this novel techniques potential to be

used during office-based bronchoscopy temporal assessment of inflammation in the time-period following intervention is possible. Additionally, as increased data is obtained correlation to clinical data such as dilation interval and tracheostomy dependence has the potential reveal a biopredictive marker capable of prognostic or diagnostic use. For example, in our cohort we identified a male patient with iSGS due to negative autoimmune serology and no history of intubation. Male iSGS patients are unusual given that iSGS is near uniformly a group comprised of middle-age Caucasian females. Interestingly, when looking at this individuals brush-biopsy cytokine profile there was not a robust increase in INF- γ gene expression as was observed in the rest of this cohort and potentially indicates a different disease status for this patient. While this is solely speculation at this point and further testing is needed to interrogate INF- γ 's role as a biomarker for iSGS, it does reinforce that diagnose is not always clear in LTS and that there is a need for ancillary testing that can aide in diagnosis.

While the gene expression of many key cytokines and immunological markers can be assessed using this minimally invasive brush biopsy technique its does have limitations. This brush-biopsy technique currently does not allow for accurate quantification of secreted cytokines or other proteins. The importance of reliable quantification of soluble cytokines confirms gene expression data and provides comprehensive insight into how changes in immune signaling affect the microenvironment of LTS scar. A potential alternative technique for endolaryngeal cytokine sampling is the use of gel foam sponges to collect surface secretions in the area of disease.^{24,25} While this established endolaryngeal technique is applicable to LTS, it is not applicable to an office-based setting and secretion analysis only reflects surface cytokines and does not garner insight into the immune response in the subepithelial layers. Additionally, it should be recognized that the data generated in this study is based on a relatively small sample size with large confidence intervals that indicated wide variability between patients. To increase the reliability of the data presented continued investigation of a larger cohort is needed to confirm these preliminary findings. Moreover, the inclusion of an additional control group of patients without LTS would be beneficial. Given this, continued optimization of our endolaryngeal sampling method to allow for quantification of soluble cytokines as well as testing in a larger cohort is needed to further improve our understanding of the inflammatory changes underlying this disease.

Conclusions

Endolaryngeal brush biopsy is a minimally traumatic and feasible approach for assessing gene expression of the immune microenvironment in LTS. This methods use as an office-based method could potentially allow for temporal assessment of inflammation in LTS and lead to identification of a novel biomarker capable of determining disease severity and prognostication. An improved method for measurement of secreted cytokines is needed, as it would improve the reliability of this sampling method. The data from this study indicates increased expression of IL-4 in idiopathic and iatrogenic LTS samples, and increased expression of INF- γ in idiopathic LTS samples. The difference in the expression of INF- γ between iSGS and iLTS further promotes the idea that different underlying mechanisms influence these histologically similar diseases. Additionally, INF- γ could represent a

potential biomarker for iSGS but these results need to be confirmed at the protein level and continued investigation into the role of these cytokines is needed.

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Figure 1. Immune and Fibrosis-related Gene Expression in Endolaryngeal LTS Brush Biopsy Samples

Expression profiles of immune and fibrosis related genes in LTS scar and normal appearing airway for seventeen LTS patients. A. Collagen-1 B. Interleukin-4 C. Interferon- γ D. Transforming growth factor- β . Error bars represent 95% confidence interval.

Figure 2. Immune and Fibrosis-related Gene Expression in iSGS and iLTS Endolaryngeal Brush Biopsy Samples

Expression profiles of immune and fibrosis related genes from LTS scar and normal appearing airway in iSGS and iLTS. A. Collagen-1 B. Interleukin-4 C. Interferon- γ D. Transforming growth factor- β . Error bars represent 95% confidence interval.

Table 1

Primer Sequences of Genes.

Gene of Interest	Primer Sequence
Interleukin-4	Forward: CCA ACT GCT TCC CCC TCT G Reverse: TCT GTT ACG GTC AAC TCG GTG
Interferon- γ	Forward: TCG GTA ACT GAC TTG AAT GTC CA Reverse: TCG CTT CCC TGT TTT AGC TGC
Transforming Growth Factor- β	Forward: GGC CAG ATC CTG TCC AAG C Reverse: GTG GGT TTC CAC CAT TAG CAC
Collagen-1	Forward: GAG GGC CAA GAC GAA GAC ATC Reverse: CAG ATC ACG TCA TCG CAC AAC
β -Actin	Forward: CAT GTA CGT TGC TAT CCA GGC Reverse: CTC CTT AAT GTC ACG CAC GAT

Table 2Demographic Data of Study Patients.^a

	<i>Total (n=17)</i>	<i>Iatrogenic (n=9)</i>	<i>Idiopathic (n=8)</i>	<i>p-value</i>
<i>Age (years)</i>	43.8	46.8	37.8	0.36
<i>Sex</i>				
<i>Male</i>	1 (6%)	0	1 (12.5%)	0.47
<i>Female</i>	16 (94%)	9 (100%)	7(87.5%)	
<i>Race</i>				
<i>Caucasian</i>	14(82.1%)	7 (77%)	8 (100%)	0.47
<i>Black</i>	2 (12%)	2 (22%)	0	
<i>Asian</i>	1 (5.9%)	1 (11%)	0	
<i>Tracheostomy</i>				
<i>Yes</i>	5 (29.4%)	5 (55%)	0	0.03
<i>No</i>	12 (70.6%)	4 (45%)	8 (100%)	

^aValues are presented as n (%) unless noted otherwise.