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Idiopathic Subglottic Stenosis is Associated with Activation of the Inflammatory IL-17A/IL23 axis:

North American Airway Collaborative (NoAAC) TS-03 study

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Abstract

Rationale—Idiopathic subglottic stenosis (iSGS) is a rare and devastating extrathoracic obstruction involving the lower laryngeal and upper tracheal airway. It arises without known antecedent injury or associated disease process. Persistent mucosal inflammation and a localized fibrotic response are hallmarks of the disease. Despite the initial clinical description of iSGS more than 40 year ago, there have been no substantive investigations into the pathogenesis of this enigmatic and progressive airway obstruction.

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Author Contribution: AG designed & performed experiments, analyzed data, & wrote the paper, NK analyzed data, MM designed & performed experiments, DN designed & performed experiments, BR aided in experimental design, JD, EE, DE, JK, AH analyzed data, preformed critical scientific review, GG aided in experimental design, LY analyzed data, JR conducted experiments, JN aided in experimental design, CW aided in experimental design, DF aided in experimental design, statistical analysis, CS conducted experiments, KJ conducted experiments, TM aided in experimental design, data analysis, review of manuscript, TB aided in experimental design, experiments, data analysis, review of manuscript, JG conducted experiments, WD aided in experimental design, experiments, data analysis, review of manuscript. Competing Interests: None of the others has any competing interest to disclose.

Objectives—In these studies, we present the initial characterization of the molecular pathogenesis underlying the fibrosing phenotype of iSGS.

Methods—Utilizing 20 human iSGS and healthy control specimens we applied histologic, immunohistochemical, molecular and immunologic techniques.

Main Results—We demonstrate significant activation of the canonical IL-23/IL-17A pathway in the tracheal mucosa of iSGS patients, as well as identify $\gamma\delta$ T cells as the primary cellular source of IL-17A.

Conclusions—Our results suggest that aberrant mucosal immune activation is a component in of the pathogenesis of iSGS. Most critically, our work offers new targets for future therapeutic intervention.

Level of Evidence—NA

Keywords

IL-17A; IL-17; Idiopathic Subglottis Stenosis; Tracheal Stenosis; Laryngotracheal Stenosis; iSGS; ISS

Introduction

Laryngotracheal stenosis is a devastating, fixed extrathoracic obstruction that can occur without known antecedent injury (idiopathic subglottic stenosis; iSGS). It also can accompany collagen vascular disease e.g. Wegeners Granulomatosis) or follow iatrogenic injury (e.g., endotracheal intubation; iLTS). iSGS is clinically rare and histopathologically distinct and manifests as persistent mucosal inflammation with a localized fibrotic tissue response. Despite the initial clinical description of iSGS more than 40 years ago¹, there have been no substantive investigations into the pathogenesis of this enigmatic and progressive airway obstruction. A recent international multicenter study (NoAAC RP-01)² demonstrated that the iSGS population is strikingly homogeneous (female, Caucasian, and perimenopausal). Classically, iSGS patients present with unexplained prodromal dyspnea (often extending several month or years prior to presentation). Physical examination demonstrates a short (1-3cm), concentric stenosis centered at the cricoid cartilage (below the level of the vocal cords). Patients require urgent treatment to reestablish airway patency. Unfortunately, surgical interventions are rarely definitive and many iSGS patients require several treatments³. High disease recidivism is common and associated with disabling side effects in breathing and voicing that significantly affect the patient's quality of life⁴.

Histologically, the majority of iSGS cases show extensive fibrosis with relatively normal cartilage⁵. Diverse diseases in divergent organ systems are associated with fibrosis, suggesting common pathogenic pathways⁶. The process is defined by the excessive accumulation of fibrous connective tissue components of the extracellular matrix (ECM, i.e. collagen and fibronectin) in inflamed tissue⁷. Despite an increasing understanding of the pathogenic mechanisms of fibrosis in alternate organ systems, there have been no studies in iSGS delineating the the biologic process driving the characteristic tissue remodeling in the subglottis.

We utilized specialized histologic staining to anatomically define the aberrant tissue remodeling in iSGS. We then employed electron microscopy to probe these changes in greater structural detail. Real-time polymerase chain reaction (RT-PCR) was performed to verify the molecular underpinnings to the observed ultrastructural fibrotic changes in iSGS. Since persistent inflammation is linked to fibrosis⁶, and inflammation in the tracheal scar is a cardinal feature of iSGS, we then investigated central immunologic mechanisms involved in inflammation that we hypothesize promote the fibrosing phenotype of this disease.

Experimental procedures

This study was performed in accordance with the Declaration of Helsinki, Good Clinical Practice, and was approved by the Institutional Review Board at Vanderbilt University Medical Center.

Patients

20 iSGS, 20 iLTS, and 23 normal controls were utilized for experiments. Each iSGS & iLTS diagnosis was confirmed using previously described clinical and serologic criteria⁸. Subglottic scar was the source of all specimens from the iSGS, and iLTS patients. The control population consisted of patients without known subglottic pathology or systemic infection.

Transmission Electron Microscopy

Human tissue samples from cases and controls were harvested in the operating theater and immediately fixed with chilled buffer (50 mM sodium cacodylate [pH 7.4]) containing 2.5% glutaraldehyde and 2.0% paraformaldehyde and placed in 4°C overnight. The samples were then prepared as previously described⁹. Samples analyzed with an FEI T-12 transmission electron microscope equipped with a side-mounted digital camera.

Immunohistochemistry

Staining—3 µm paraffin embedded sections of iSGS or iLTS airway stenosis derived from patients that underwent open tracheal or cricotracheal resection were obtained from the Vanderbilt pathology archive. Normal subglottis from healthy controls was obtained from US Biomax Inc. (product# RS321). Specimens were deparaffinized, and a heat induced antigen retrieval was performed for 20 minutes. Slides were placed in DakoCytomation Biotin Blocking System (Ref#x0590, DAKO) for 15 minutes in each solution. Slides then were placed in a Protein Block (Ref# x0909, DAKO) for 10 minutes. Slides were incubated with anti-IL-17A (Cat. AF-317-NA, R&D Systems) for one hour at a 1:200 dilution and followed by a biotinylated anti-goat (Cat. BA-5000, Vector Laboratories, Inc.) for 30 minutes at a 1:200 dilution. The Bond Polymer Refine detection system was used for visualization. Slides were then dehydrated, cleared and coverslipped.

Quantification—Immunostained slides were imaged on a Leica SCN400 Slide Scanner (Leica Biosystems). Slides were imaged at 20X magnification to a resolution of 0.5 µm/pixel. Cells were identified utilizing standard Ariol® analysis scripts. (Leica) Upper and lower thresholds for color, saturation, intensity, size, roundness, and axis length were set for

both blue Hematoxylin staining of nuclei and for brown DAB reaction products. Thus, brown (DAB) positive cells can be distinguished from blue (Hematoxylin only) negative cells.

qPCR profiling of respiratory epithelial immune activation

Following mechanical digestion of surgical biopsy specimens, mRNA was extracted with the RNeasy extraction kit (Qiagen, Valencia, CA) according to the manufacturer's instructions. Healthy control tracheal mRNA was obtained from 23 pooled donors (clontech cat# 636127)¹⁰. The RNA concentration and quality were confirmed using the Bioanalyzer 2100 system (Agilent, CA, USA). cDNA was transcribed using established reagents according to manufacturer's instructions. The human innate and adaptive immune response qPCR array (Qiagen) was utilized as per manufacture's instructions in a StepOnePlus™ instrument (Applied Biosystems®, USA). Expression analysis was preformed using PCR analysis software employing the 2-^{-Ct} method as previously described¹¹ (Qiagen; <https://www.qiagen.com>).

Flow Cytometry

Surgical specimens of tracheal scar were digested and single cell suspensions were rested overnight in RPMI 1640 medium supplemented with 10% FCS. 5×10^5 cells were activated with PMA and ionomycin at 37 °C under 5 % CO₂ for 30 min before addition of BD GolgiStop (BD Biosciences). After 4h of stimulation, the cells were harvested and washed for surface staining to CD3, TCR $\gamma\delta$, (BD Biosciences). Cells were then washed, fixed and permeabilized, using a Fix & Perm Kit according to the manufacturer's instructions (BD Biosciences). Intracellular antibodies to IL-17A were added at 4 °C for 45 min. Cells were washed and analyzed via flow cytometry. For the gating strategy, lymphocytes were gated by FSC/SSC, then doublets were gated out, then dead cells, then cells were analyzed for CD3 and SSC. CD3+ cells were gated and subsequently analyzed for $\gamma\delta$ TCR and IL-17A expression. All flow cytometry experiments were acquired with a LSR-II flow cytometer (BD Biosciences). Analysis was performed using FlowJo software (Tree Star). A minimum of 200,000 events were acquired for each sample.

Statistical Analysis

Statistical significance was set at p value less than 0.05 and a mean difference equal to or greater than 2-fold change in expression levels. Normal distribution of the variables was tested using the Shapiro-Wilk test. Differences between x and y groups were determined using the Kruskal Wallis and Mann Whitney tests for normal and non-normal distributions, respectively. Data were expressed as median $\pm$ SD for non-normal distributed variables. All statistical analyses were performed with Prism version 6.0 software.

Results

iSGS Anatomic, Physiologic and Histologic characteristics

iSGS is defined by classic anatomic, physiologic and histologic characteristics. All iSGS patients develop airway stenosis below the vocal cords in the region where the larynx transitions to the trachea (Fig. S1A., S1B.). This luminal scar produces impairment in

airflow manifesting as a flow-volume loop finding of fixed extrathoracic obstruction on pulmonary function testing (PFT), (Fig. S1C.). Histologically, the airway mucosa at the site of scar is frequently characterized by an intact basement membrane (Fig. 1A.) with pronounced subepithelial fibrosis (Fig. 1B.). Transmission electron microscopy demonstrates the disordered arrangement of collagen bundles (Fig. 1C.) driven by abundant tissue fibroblasts (Fig. 1D.). Molecular analysis of areas of subglottic fibrotic remodeling shows increased mRNA expression of established extracellular matrix constituents type I collagen (COL1A2), type III collagen (COL3A1), and Fibronectin (FN1) (Fig. 1E.). These results were confirmed at the protein level with histologic analysis (data not shown).

Host Inflammatory Response

Affected tracheal mucosa in iSGS patients consistently demonstrates acute and chronic inflammation (Fig. S2A), with an infiltration of CD3+ T lymphocytes (Fig. S2B). To delineate the host mucosal immune response in iSGS, we utilized a qPCR array consisting of 84 unique inflammatory genes (Qiagen Inc, Vallengia, CA) to compare gene expression in the tracheal mucosa of 10 iSGS patients with 23 healthy controls (Fig. S3). mRNA expression analysis showed the IL-17A to be the most significantly unregulated gene in iSGS mucosa compared to healthy control trachea ($p < 0.005$) (Fig. 2A). The upstream driver of the IL-17A pathway, IL-23 demonstrated significant up-regulation in iSGS and IL-1 β showed a trend towards increased expression, suggesting a potential role for the IL-17A/IL-23 pathway in the pathogenesis of inflammation and fibrosis seen in iSGS.

Increased IL-17A expression within the mucosa of iSGS scar was confirmed at the protein level utilizing immunohistochemistry. As seen in the representative images (Fig. 2B) and quantified digitally by computerized algorithm, the mean number of IL-17A positive cells in iSGS tracheal mucosal biopsy specimens was 30.3% compared to 8.1% in iLTS (who developed their tracheal injury following prolonged endotracheal intubation in an ICU for medical illness) and 1.3% in normal healthy tracheal mucosa; $p < 0.001$].

In order to localize the cellular source of mucosal IL-17A, we utilized flow cytometry with intracellular cytokine staining on cells obtained from surgical specimens containing subglottic mucosal scar tissue from 3 iSGS patients. After digestion and nonspecific stimulation, Gr1⁺neutrophils, CD3⁺CD4⁺, and CD3⁺ $\gamma\delta$ TCR⁺ positive cells were assessed for intracellular IL-17A levels. In iSGS, 87-97% of IL-17A expressing cells were identified as $\gamma\delta$ TCR⁺(Fig. 2C).

Discussion

Although the clinical description of iSGS is more than 40 years old¹, our study represents the first molecular characterization of the pathogenic tissue remodeling in this rare disorder. The in-depth histologic, electron microscopic, and molecular techniques utilized in this study confirm an abundance of ECM components in the subglottic scar in iSGS patients. This evidence strongly supports a central role for the pathogenic process of fibrosis in the tissue remodeling seen in iSGS. Furthermore, our studies highlight the inflammatory nature of the airway scar in iSGS, and begin to characterize the immunologic mechanisms driving the observed inflammation.

While acute and chronic inflammation are characteristic clinical findings in the subglottic scar of iSGS patients, our study begins to dissect the molecular circuits involved. Our data reveals a significant up-regulation of the IL-17A/IL23 inflammatory axis in iSGS airway scar. Although crucial in protecting the host from invasion by many types of pathogens, dysregulated IL-17A production can drive chronic inflammation¹²⁻¹⁴ with subsequent tissue damage and fibrinogenesis¹⁵¹⁶. IL-17A is also specifically implicated in many inflammatory lung diseases, including asthma¹²¹⁷, cystic fibrosis¹³ and chronic obstructive pulmonary disease¹⁴. Classically, the expression of IL-17A is derived from immune cells located in non-lymphoid tissues where they are poised to respond immediately to tissue injury or pathogenic insults. Stimulation of these cells by IL-23 induces IL-17A expression and subsequent local tissue inflammation. Consistent with established mechanistic work, our investigations reveal an increase in IL-23 accompanying upregulated IL-17A expression within an infiltrating immune cell subset in the subglottis.

Several studies in the last 5 years have linked IL-17A and the development of fibrotic lung disease. Human studies offer strong support for a critical role of IL-17A in the pathogenesis of obliterative bronchiolitis¹⁶ seen after pulmonary transplant. However, the mechanisms governing IL-17A-mediated fibrosis in pulmonary fibroinflammatory disease remain poorly understood. The elevated inflammatory IL-17A expression we observed in the subglottis may directly influence fibroblasts in iSGS leading to pathologic tissue fibrosis¹⁸, although the precise molecular mechanism linking IL-17A to fibrogenesis merits future study.

Given the rarity of iSGS, our results will require confirmation in larger cohorts pooled from multiple institutions. Furthermore, while we provide molecular characterization of the fibroinflammatory phenotype as mechanistic support for the hypothesis that a conserved and consistent biologic process drives a singular disease, the explanation for why involvement is nearly universally restricted to adult Caucasian women remains opaque. The role of genetics and the influence of estrogen on host inflammation and fibrosis are novel questions that demand future studies.

The inciting event behind the dramatic increase in IL-17A is also unclear. However, given the established role of $\gamma\delta$ T cell IL-17A in host defense against pathogens at mucosal barriers¹⁹, as well as an emerging understanding of disrupted microbiota in other chronic inflammatory mucosal disease²⁰⁻²⁶, our results raise important questions about a potential role for pathogens in the etiopathogenesis of iSGS. Given the advent of robust culture-independent techniques²⁷²⁸ future studies may be able to define a role for bacteria, fungus, or virus in disease initiation, progression, or recurrence.

Alternatively, a role for IL-17A has been increasingly recognized in autoimmune collagen vascular disorders such as rheumatoid arthritis (RA)¹⁸, systemic lupus erythematosus (SLE)²⁹, and granulomatosis with polyangiitis (GPA, i.e. Wegener's granulomatosis)³⁰³¹. In these diseases, increased circulating IL-17A⁺ cells have been closely associated with tissue inflammation and overall disease activity. Our findings may offer support for the phenotypic similarity between iSGS and GPA observed clinically. However, future study will be important to full clarify the molecular overlap³¹ between the diseases.

Our results advance the understanding of iSGS by developing the concept that this disease results from aberrant mucosal immune activation in the large airway rather than simply idiopathic extracellular matrix deposition. Most critically, demonstration of IL-23/IL-17A pathway activation offers a novel approach for therapeutic intervention in iSGS patients. Several established drugs limit IL-17A production³², or neutralize its effects^{33,34}. Future insight into the molecular mechanism linking IL-17A to canonical pathways of tissue fibrosis may reveal additional pathways amenable to directed targeting. Thus, the implications this work may extend beyond the confines of iSGS to impact other fibrotic diseases driven by pathogenic inflammation.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. iSGS Anatomic, Physiologic and Histologic characteristics

Trichome blue staining of iSGS subglottic scar demonstrating extensive blue collagen staining and an intact basement membrane (Magnification 20x, scale bar = 100 μ M) (A.). Sirius red staining highlights subepithelial collagen deposition in red (Magnification 40x, scale bar = 100 μ M) (B.). Transmission electron microscopy shows irregular, disordered subepithelial collagen bundles (C.), as well as abundant fibroblasts (D.). qPCR from 10 iSGS patients for extracellular matrix constituents (Types I & III collagen, Fibronectin) from iSGS scar compared with 23 healthy controls (E.), asterisk indicates statistical significance (Two-tailed, Mann Whitney test; $p < 0.05$).

Figure 2. Host Inflammatory Response

qPCR results for panel IL-17A/IL-23 pathway gene products from 10 iSGS patients demonstrating significant upregulation in IL-17A and IL-23 when compared to control healthy trachea, asterisk denotes significance (Two-tailed, Mann Whitney test; $p < 0.005$). IL-1 β tended to be higher in iSGS patients but did not reach statistical significance ($p = 0.06$) (A.). Representative images from IHC of IL-17A in surgical specimens from iSGS ($n = 10$), iLTS ($n = 10$), and control ($n = 10$) patients (Magnification 20x, scale bar = 100 μ M). Accompanying graph summarizing digitally quantified IL-17A levels, depicting significantly more IL-17A expression in iSGS patients, compared with either iLTS, or controls, asterisk denotes significance (Kruskal-Wallis test; $p < 0.0001$) (B.). Flow cytometry from tracheal mucosa in 3 surgical specimens from different iSGS patients, demonstrating that the majority of IL-17A positive cells were $\gamma\delta$ TCR positive (C.).