Pulse Steroid Therapy Inhibits Murine Subglottic Granulation

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Sponsorships or competing interests that may be relevant to content are disclosed at the end of this article.

Abstract

Objective. Using a functional model of airway granulation tissue in subglottic stenosis, we investigated changes in inflammatory markers within granulation tissue in response to intraperitoneal dexamethasone injections. Changes in inflammatory markers will allow us to identify potential targets for immunological therapy.

Study Design. Institutional Animal Care and Use Committee–approved animal study.

Setting. Philadelphia Veterans Administration Medical Center animal research facility.

Subjects and Methods. Laryngotracheal complexes of donor mice underwent direct airway injury and were transplanted into subcutaneous tissue of 19 recipient mice in 2 groups: steroid treated and untreated, with sample sizes of 10 and 9, respectively. The steroid-treated arm received intraperitoneal injection of dexamethasone for 3 weeks. Laryngotracheal complexes were then harvested, and granulation formation was measured. The messenger RNA (mRNA) expression of transforming growth factor (TGF)–β1 and interleukin (IL)–1β was quantified.

Results. At 3 weeks posttransplantation, there were statistically significant differences in observable granulation formation as well as mRNA expression of TGF-β1 and IL-1β in all groups within the steroid treated arm as compared with the untreated arm.

Conclusions. Systemic steroids have been used to prevent formation of granulation tissue and subglottic stenosis. However, the study of the immunologic markers and the corresponding changes with steroid treatment has not been well studied in animal models. Using a previously described novel murine model, we begin to delineate inflammatory markers that can be applied for potential therapeutic targets.

Keywords

subglottic stenosis, granulation tissue, murine model, steroids

Background

Subglottic stenosis is the result of abnormal wound-healing processes leading to hypertrophic scar formation and obstruction of the airway lumen by excess granulation tissue. Furthermore, wound healing is a dynamic and complex process mediated by a wide range of coordinated cellular reactions, which can be influenced by both local and systemic parameters, such as infection, pressure, tissue necrosis, age, and other patient comorbidities. The most widely practiced treatments include systemic antibiotics, systemic steroids, hyperbaric oxygen, anti-inflammatory agents, antireflux therapy, and, finally, open or endoscopic surgical repair. Understanding the pathologic wound-healing process may help find methods to treat and prevent subglottic stenosis.

Precise cellular and molecular processes underlying fundamental aspects of upper airway injury, inflammation, and fibrosis remain poorly defined. To understand the wound-healing pathways, it is necessary to comprehend the temporal pattern of inflammatory mediators that lead to migration of fibroblasts and ultimate granulation tissue formation. Several types of mucosal injury have demonstrated the clinical utility of measuring biochemical markers in secretions. Tissues of the intestine have been analyzed for the presence of factors affecting healing and fibrosis to document the wound-healing process, further confirming the clinical utility of measuring biochemical markers in secretions. Several types of mucosal injury have demonstrated the clinical utility of measuring biochemical markers in secretions. Tissues of the intestine have been analyzed for the presence of factors affecting healing and fibrosis to document the wound-healing process, further confirming the potential of such assays. Furthermore, secretion analysis has been extended to wound healing in the upper airway to understand the process of wound healing in subglottic stenosis. It has been reported that patients with vocal fold lesions present differing profiles...

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of wound-healing markers compared with normal subjects.\(^4\)

It has thus been hypothesized that those patients who develop stenosis of the airway will present with a distinctly different inflammatory response and, therefore, a different profile of inflammatory mediators. This paragraph discusses the temporal expression, implying that certain markers are expressed at different times. However, we are studying which markers are expressed and how much, not when.

A number of inflammatory markers (ie, cytokines) have been implicated in the development of airway stenosis. It has become imperative to characterize the role of such mediators in the development of granulation tissue. The major cytokine observed in chronic inflammatory tissue has been transforming growth factor–β\(_1\) (TGF-β\(_1\)); Smad markers have been linked to the TGF-β\(_1\) granulation pathway.\(^5\) Similarly, interleukin-1 (IL-1) has been shown to be present in high concentrations in patients with subglottic stenosis.\(^6\) Given the previous implications, we chose to focus our experiment mainly on these markers.

This study was designed to address the following questions: (1) Does epithelial injury to upper airway mucosa result in the upregulation of implicated inflammatory genes? (2) Will injecting dexamethasone intraperitonally, a known therapy for laryngotracheal stenosis, change levels of interleukins and TGF-β compared with the untreated control subjects?

The current study is an extension of previous work, using the ex vivo murine model of subglottic granulation developed by our lab to study messenger RNA (mRNA) expression levels of an array of inflammatory markers through reverse transcription–polymerase chain reaction (RT-PCR) and to further understand the inflammatory response that leads to the development of subglottic stenosis. We hypothesize that we will find measurable differences in mRNA expression levels of IL-1 and TGF-β between injured and uninjured trachea. In addition, we expect to find that pulse steroid therapy yields statistically significant differences in the mRNA expression of these markers in the steroid-treated mice compared with untreated mice. With this information, we will be able to understand the inflammatory process behind subglottic stenosis and develop therapeutic regimens aimed specifically at inhibiting the inflammatory markers that lead to the granulation tissue causing the pathology.

**Materials and Methods**

**Experimental Design**

Laryngotracheal complex (LTC) transplants were performed using donor airway segments from 5- to 7-week-old, 20- to 25-g C57BL/6 mice (Jackson Laboratories, Bar Harbor, Maine). Laryngotracheal complexes from 76 donor mice were harvested and divided into three groups: (1) uninjured, (2) mechanical injury using a wire brush, and (3) chemical injury using hydrochloric acid (HCl). The C57BL/6 donor segments were transplanted into the deep subcutaneous tissue of 19 recipient wild-type C57BL6 mice (Jackson Laboratories). 1 donor LTC from each group was placed into deep dorsal subcutaneous pockets of recipient C57BL/6 mice, for a total of 3 transplanted per recipient mouse (Figure 1). Mice in the steroid arm only (10 recipient mice marked by ear tags) then underwent pulse steroid therapy with dexamethasone for a 3-week period. Recipient mice were monitored daily for evidence of infection or extrusion of transplanted donor segments.

All animal studies were approved by and performed in accordance with Institutional Animal Care and Use Committee (IACUC) of the Philadelphia Veterans Affairs Medical Center guidelines. All C57BL6 mice were housed in an Association for Assessment of Laboratory Care–approved facility in a pathogen-free environment with climate-controlled rooms and free access to standard pelleted food and sterile water. Sample size was calculated via Mead’s resource equation for laboratory animals, where \(E = N - B - T\), where \(E\) is the degrees of freedom of the error component and should be somewhere between 10 and 20. Given that \(T\) (the treatment component, corresponding to the number of treatment groups) was 6 total and \(B\) (the blocking component, representing environmental effects allowed for in the design) was 0, this amounted to at least 4 mice from each treatment group for PCR values.

**LTC Transplantation**

Donor C57BL/6 mice were euthanized using a compressed carbon dioxide (CO\(_2\)) chamber. Once mice were confirmed dead, a vertical mental to sternal incision was made and the
LTC exposed through careful dissection. The uninjured group served as controls with direct harvesting and transplanting of the LTCs. The LTCs in the injured group were kept in situ and exposed to direct subglottic insult through 1 of 2 methods: mechanical or chemical injury. The mechanical injury group underwent direct mucosal scraping by passing a wire brush with a diameter of 0.007 inches through the inside of the trachea a total of 15 times through a pharyngotomy incision, thus abrading the subglottic mucosa. The chemical injury group underwent injection to the subglottic mucosa of 0.5 mL HCl titrated to a pH of 4. After 5 minutes, the acid was irrigated out of the airway using a saline injection. Uninjured and injured LTCs were excised and placed in saline to await transplantation into the recipient mouse.

Recipient mice were weighed and anesthetized with an intraperitoneal injection of ketamine (80 mg/kg) and xylazine (16 mg/kg). Once anesthetized, the mice were shaved along the dorsum and hindside. After surgical preparation using a sterile technique, 0.5-cm horizontal incisions were made on the left and right dorsum and on the hindside. Connective tissue was pulled back to create 3 deep subcutaneous pockets, into which an LTC from the control group and each experimental group was placed (Figure 2). After each LTC was placed in the appropriate pocket, incisions were carefully closed with Dermabond (Ethicon, Somerville, New Jersey). The recipient mouse was then placed on a warmer and observed until it regained consciousness.

Recipient mice were treated with oral acetaminophen for postoperative pain and monitored daily for signs of infection.

Steroid Therapy
Over a 3-week course, mice in the treatment arm were intraperitoneally injected the glucocorticoid steroid dexamethasone at a dosage of 0.5 mg/kg; for a 25-g mouse, 0.0125 mg of dexamethasone would be administered. To produce a volume measurable in a 1-mL syringe, a concentrated solution of dexamethasone (10 mg/mL) was diluted with saline to produce a diluted steroid solution of 0.5 mg/mL. For each C57BL6 mouse of approximately 25 g, 0.025 mL of dilute solution was injected every other day.Recipient mice were monitored daily for evidence of infection or extrusion of transplanted donor segments.

At 3 weeks after surgery, LTCs were harvested from all mice (treated and untreated). This was done by euthanizing recipient mice in a CO2 chamber and opening the previously made incisions to carefully dissect out each transplanted specimen (Figure 3).

RT-PCR
The primers used for real time RT-PCR were IL-1 and TGF-β. Messenger RNA collected and extracted from harvested tissues was reverse transcribed into complementary DNA (cDNA) and quantitatively amplified using RT-PCR. The reverse transcriptase reaction included 1 μg DNA-free total RNA pooled from each group, 1 μL oligo-(dT), and a DNA polymerase (Superscript III; Invitrogen, Carlsbad, California) and was incubated at 65°C for 5 minutes, 50°C for 45 minutes, and 70°C for 15 minutes in a thermocycler (PE 2700; Applied Biosystems, Foster City, California). The RT-PCR reaction included the SYBR Green Premix Kit (Applied Biosystems), 0.5 μL of each gene-specific forward and reverse primer (5 μM), and 6 μL of cDNA in a final volume of 50 μL. The conditions for real-time PCR were as follows: 50°C for 2 minutes, 95°C for 12 minutes, 40 cycles at 95°C for 15 seconds, and 60°C for 1 minute in a sequence detection system (ABI PRISM 7700; Applied Biosystems). The system-specific sequence detection software (Applied Biosystems) was used for instrument control.
automated data collection, and data analysis. Relative quantitation (fold difference) of the expression levels of each transcript for each group was calculated by means of the $2^{-\Delta\Delta CT}$ method, producing a value inversely related to the relative abundance of the mRNA. Values were then standardized to mRNA values derived from control tissue obtained as described earlier and expressed as fold increase.

Analyses

The LTC specimens from control, chemical injury, and mechanical injury groups were harvested from 10 steroid-treated and 9 control C57BL recipient mice. From the steroid group, 7 sets of specimens were used for quantitative PCR analysis, and 3 sets were used for hematoxylin and eosin staining. From the control group, 5 sets of specimens were used for quantitative PCR analysis, and 4 sets were used for hematoxylin and eosin staining (one of which was thrown out for poor sectioning).

For quantitative PCR analysis, upregulation of inflammatory marker genes is expressed as fold increase above levels in control tissue, after normalization to an internal control gene. Data are expressed as averages. Steroid-treated data values were then compared with untreated data values using a nonparametric Student $t$ test for statistical analyses.

Results

Histologic Analysis

In the untreated chemically and mechanically injured specimens ($n = 4$), there was evidence of 25% to 75% obstruction of the tracheal lumen with granulation tissue in the lamina propria beneath a notably attenuated airway epithelium (Figure 4). Subepithelial granulation was demonstrated by the presence of fibroblasts, angiogenesis, and inflammation with a predominantly lymphocytic infiltrate. Of note, granulation tissue was not circumferentially uniform; rather, it developed in a focal pattern. The untreated control trachea, in contrast, demonstrated preserved epithelium and limited cellular infiltrate.

In marked contrast, all specimens taken from steroid-treated chemically and mechanically injured specimens showed both attenuated and regenerated epithelium with little to no evidence of granulation, with a marked absence of cellular infiltrate (Figure 5). The treated control trachea demonstrated preserved epithelium and an absence of cellular infiltrate.

mRNA Expression

At 3 weeks posttransplantation, there were statistically significant differences in observable granulation formation as
well as mRNA expression of TGF-β1 and IL-1β in both chemically and mechanically injured groups within the steroid-treated arm as compared with the untreated arm. Because the uninjured trachea in each group was used as control for the quantitative PCR calculation (please refer to Materials and Methods section), mRNA expression is designated as fold increase above control.

Using quantitative PCR (SYBR Green), we found that mRNA expression of TGF-β1 and IL-1β levels was upregulated compared with control trachea in all injured trachea, implicating that an inflammatory reaction had occurred in response to epithelial injury. Furthermore, we observed that mRNA expression of TGF-β1 and IL-1β levels in steroid-treated tissues was significantly decreased compared with the untreated arm. Control values used in the calculation of fold change in expression for each trial were obtained by amplifying complementary DNA obtained from transplanted control airways. Control transplanted tracheas were chosen because they contain an elevated inflammatory response relative to native trachea, which is induced by the trauma of transplantation, as evidenced by previous work in the lab that showed slightly increased levels of inflammatory markers (1.5-fold) as well increased histologic evidence of inflammation compared with native trachea.

Compared with values from the untreated arm, IL-1β levels in the steroid-treated subglottic mucosa decreased at the harvest time of 3 weeks postinjury. Interleukin-1β values decreased from approximately 21- to 3-fold in the chemical injury group and 3- to 2-fold in the mechanical injury group (Table 1 and Figure 6). All values were significant, with P values less than .05.

Transforming growth factor-β1 levels also decreased, from approximately 3- to 0.5-fold in both the chemical and mechanical injury groups (Table 1 and Figure 7). All values were significant, with P values less than .05.

### Discussion

Wound healing is regulated by growth factors, cytokines, chemokines, and other inflammatory mediators. Changes in the inflammatory profile have been associated with significant alterations in the overall outcome of wound healing, specifically to the development of fibrotic conditions. It is thus imperative to describe the expression of inflammatory mediators in aberrant wound healing and ultimate fibrosis. To date,

### Table 1. Average Data Values

<table>
<thead>
<tr>
<th></th>
<th>Mechanical Untreated</th>
<th>Mechanical Treated</th>
<th>P Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>TGF-β</td>
<td>2.1333 (1.79-2.52)</td>
<td>0.395 (0.039-0.41)</td>
<td>.0013</td>
</tr>
<tr>
<td></td>
<td>[0.1910-2.3060]</td>
<td>[0.193-1.268]</td>
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</tr>
<tr>
<td>IL-1</td>
<td>3.435 (2.21-5.88)</td>
<td>0.36876 (0.031-0.992)</td>
<td>.012</td>
</tr>
<tr>
<td></td>
<td>[0.9546-6.2832]</td>
<td>[0.242-1.594]</td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th></th>
<th>Chemical Untreated</th>
<th>Chemical Treated</th>
<th>P Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>TGF-β</td>
<td>3.17 (2.63-3.59)</td>
<td>0.124 (0.011-0.107)</td>
<td>&gt;.001</td>
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<tr>
<td></td>
<td>[0.2557-3.0866]</td>
<td>[0.089-0.589]</td>
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</tr>
<tr>
<td>IL-1</td>
<td>21.2667 (18.96-23.27)</td>
<td>0.04538 (0.024-0.065)</td>
<td>&gt;.001</td>
</tr>
<tr>
<td></td>
<td>[1.066-7.017]</td>
<td>[0.025-0.166]</td>
<td></td>
</tr>
</tbody>
</table>

Values are presented as No. (range) [confidence interval]. Abbreviations: IL-1, interleukin-1; TGF-β, transforming growth factor–β.

### Figure 6.
Treated vs untreated values of inflammatory markers in chemical injury specimens. Note that there is a significant and measurable difference in the values of transforming growth factor–β (TGF-β) and most especially in interleukin-1 (IL-1), with n = 4.

### Figure 7.
Treated vs untreated values of inflammatory markers in mechanical injury specimens. Note that there is a significant and measurable difference in the values of transforming growth factor–β (TGF-β) and interleukin-1 (IL-1), with n = 4, that is less pronounced but still significant.
no animal model has been used specifically in obtaining tissue to develop inflammatory profiles in profibrotic pathology.

This study represents a novel approach to studying the inflammatory processes leading to subglottic stenosis through tissue analysis rather than secretion analysis. Using an animal model of subglottic granulation, we aimed to clarify the precise cellular and molecular processes underlying the development of stenosis with a view toward developing clinical tools for novel treatment to prevent the scarring process. Using our established model of murine airway injury, we undertook to study the temporal pattern of expression of implicated inflammatory markers in the resulting subglottic granulation tissue and found that there are significant, measurable, postinjury differences over time. Assessing the expression of implicated inflammatory markers in the untreated group at 1, 2, and 3 weeks posttransplantation in previous work done by our laboratory on the temporal expression of implicated inflammatory markers, we discovered that the most significant upregulation of TGF-β and IL-1, among other markers, was at the 3-week period. Thus, a 3-week treatment period was chosen from previous laboratory work to examine the effect of steroids on granulation formation and inflammatory markers. The data from this experiment and from the previous work done by our laboratory mark the use of granulation tissue obtained from the murine model of airway epithelial injury as a possible future tool to elucidate the inflammatory course of upper airway wound healing in all its stages and to test the effects of novel therapy.

Following injury, inflammatory cells invade the wound and secrete an array of early and primary inflammatory mediators, including IL-1β, which will subsequently activate downstream inflammatory signals. Interleukin-1β is an obvious target for such an experiment because its role is well defined as an early mediator of the inflammatory response. Measurable amounts of this mediator have been previously observed, shown to be highest in the early days of wound healing.

In addition, the major mediator observed in chronic inflammatory tissues, though not secretions, had been TGF-β. Previous studies have suggested TGF-β plays a central role in fibrosis by regulating the deposition of extracellular matrix (ECM) components such as collagen, fibronectin, and proteoglycans. Transforming growth factor-β1 has an effect on procollagen formation, fibroblast conversion, and ECM deposition. In fact, a murine model of subglottic stenosis has been documented in which immunohistochemical staining of granulation tissue for TGF-β was used, with the result that elevated levels of TGF-β1 have been demonstrated in the lamina propria following subglottic injury. Thus, the dominant factor in late stages of tracheal stenosis seems to be the high-level expression of TGF-β1 and the deposition of extracellular matrix. It is thus imperative to study the mRNA expression of TGF-β.

This experiment tests our previous research finding an upregulation in inflammatory markers using a known therapy for tracheal stenosis. Dexamethasone, a glucocorticoid steroid, has been shown in previous studies to inhibit TGF-β1 expression during wound healing. Research has also been conducted in alveolar cow macrophages with data supporting the inhibition of IL-1β expression by dexamethasone. These steroids activate a feedback mechanism in the immune system that turns immune activity down by binding to the glucocorticoid receptor. The activated glucocorticoid receptor complex then upregulates anti-inflammatory proteins through the transactivation process while repressing the expression of proinflammatory proteins through transrepression.

The downregulation of TGF-β and IL-1 in the steroid-treated arm compared with the untreated arm corroborates with the expected effect of steroid therapy. Transforming growth factor-β in chemically injured tissue decreased from 1-fold to 0.16-fold, whereas IL-1 upregulation decreased from 21-fold to 0.21-fold. In mechanically injured tissue, TGF-β decreased from 1.63-fold to 0.44-fold. The same injury caused IL-1 to decrease from over 2-fold to under 1-fold, indicating that perhaps dexamethasone plays a more important role in downregulating the inflammatory response produced as a reaction to chemical injury than that produced in reaction to mechanical injury. Another possibility is that the extent of acid injury is more extensive and deeper as compared with mechanical injury, eliciting a more pronounced early inflammatory reaction in the untreated arm. With pulse steroid therapy, this more pronounced reaction is inhibited.

Although steroids are frequently used, there are little published definitive data regarding the utility of such injections or the topical application of steroids. The optimal timing, route of administration, and dosing are still primarily anecdotal in the literature, and we aim to define such parameters in future studies. Primarily, this study served to define the effect of steroids on a molecular level of wound healing and to define the role of steroids in the downregulation of inflammatory markers. It is to lay the groundwork to determine optimal dosing and route of administration for future steroid use in subglottic stenosis. Also of future interest is the role of anti-inflammatory therapies earlier in the wound-healing process. This initial experiment did visualize the effect of the gold standard of steroids on implicated inflammatory markers and will serve as a marker for the comparative effect of other directed therapies.

In addition, the study of inflammatory markers in granulation tissue and the study of potential therapies provide avenues for translational research in human tissue. Because steroids have many diverse effects, including side effects that could be harmful in humans, the study requires optimization for treatment in humans. We will be conducting similar studies on human granulation tissue for similar inflammatory markers and use these potential therapies to prevent or treat subglottic stenosis. Although such an outcome to a clinical trial requires numerous additional studies, our research could provide a novel and effective treatment mechanism for this disorder.

Author Contributions
Ankona Ghosh, study design, collection of data, analysis and interpretation of the data, writing, editing; Genevieve Philippou, study design, collection of data, analysis and interpretation of the data, writing, editing; Ankona Ghosh, study design, collection of data, analysis and interpretation of the data, writing, editing; Genevieve Philippou, study design, collection of data, analysis and interpretation of the data, writing, editing.
collection of data, analysis and interpretation of the data, writing, editing; **Jennifer Y. Lee**, study design, editing; **Kevin P. Leahy**, study design, collection of data, analysis and interpretation of the data, editing, approval of the manuscript; **Sunil Singhal**, study design, editing, approval of the manuscript; **Noam A. Cohen**, study design, editing, approval of the manuscript; **Natasha Mirza**, initial idea, study design, editing, approval of the manuscript.

**Disclosures**

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**References**