Development of an Animal Model for Wound Healing in Chronic Rhinosinusitis

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Objective: To develop a model for sinonasal wounding and evaluation during healing in mice with chronic eosinophilic inflammation.

Design: Exploratory controlled study in which chronic eosinophilic nasal and sinus inflammation was established in mice followed by wounding of the sinonasal cavity. Histologic features and gene expression were then studied.

Setting: University of Utah Center for Comparative Medicine.

Subjects: Chronic eosinophilic inflammation was established in mice. They were then wounded and humanly killed at days 3, 7, and 14 after wounding.

Main Outcome Measures: Inflammation was assayed by light microscopic examination. Polymerase chain reaction analysis of transforming growth factor-B1b, insulinlike growth factor (IGF)-1, matrix metalloproteinase (MMP)-7, MMP-9, tissue inhibitor of metalloproteinase 1 (TIMP-1), and prostaglandin E receptor EP4 expression was performed as well. Uninflamed mice were wounded and examined using the same protocol.

Results: Chronically inflamed mice showed higher histologic inflammatory scores before and after wounding. Expression of IGF-1, TIMP-1, and MMP-9 was also higher prior to wounding and during healing. Continued stimulation appears necessary for the chronic eosinophilic inflammation to persist.

Conclusions: We successfully constructed a model in which wound healing in a setting of chronic eosinophilic inflammation can be studied. In this exploratory pilot, we demonstrated the feasibility of reproducibly wounding the sinonasal cavity of chronically inflamed mice and examining histologic and gene expression effects of the inflammatory response after wounding.


CHRONIC RHINOSINUSITIS AFFECTS MILLIONS OF INDIVIDUALS IN THE UNITED STATES AND ACCOUNTS FOR BILLIONS OF DOLLARS IN DIRECT MEDICAL COSTS ANNUALLY. INDIVIDUALS IN WHOM MEDICAL MANAGEMENT FAILS ARE CONSIDERED SURGICAL CANDIDATES, WITH SINUS SURGERY BEING PERFORMED NEARLY 500,000 TIMES IN THE UNITED STATES ANNUALLY IN AN ATTEMPT TO REDUCE THE BURDEN OF SINUS DISEASE. UNFORTUNATELY, SURGERY IS OFTEN COMPLICATED BY ADHESIONS AND SCARRING THAT CAN COMPROMISE THE SUCCESS OF THE PROCEDURE. DESPITE THE HIGH FREQUENCY WITH WHICH SINUS SURGERY IS PERFORMED, VERY LITTLE IS KNOWN ABOUT NORMAL WOUND HEALING PROCESSES IN CHRONICALLY INFLAMED SINUS MUCOSA. WITH THOUSANDS OF PATIENTS EACH YEAR REQUIREING REVISION SINUS SURGERY, A BETTER UNDERSTANDING OF SINUS WOUND HEALING IN A CHRONIC RHINOSINUSITIS MODEL IS NEEDED TO GUIDE TREATMENTS AND PRACTICES.

A PRINCIPAL BARRIER IN UNDERSTANDING SINUS WOUND HEALING IS AN ACCEPTABLE ANIMAL MODEL. RABBITS HAVE CHARACTERISTICALLY BEEN USED FOR SINUS EXPERIMENTS AND ARE APPLICABLE AS A MODEL FOR ACUTE BACTERIAL SINDUSITIS, BUT A CHRONIC NONINFECTIONOUS INFLAMMATION IS DIFFICULT TO MAINTAIN IN THIS MODEL. THE INFLAMMATORY RESPONSE IN RABBITS IS DIFFERENT FROM HUMANS, AND THERE ARE FEW REAGENTS AND GENETICALLY MODIFIED SPECIES AVAILABLE. SHEEP HAVE ALSO BEEN PROPOSED BECAUSE OF THEIR ABILITY TO DEVELOP EOSINOPHILIC INFLAMMATION, BUT THEIR COST AND LACK OF GENETIC ALTERATIONS ARE ALSO PROHIBITIVE.

RECENTLY, A MURINE MODEL OF CHRONIC NONINFECTIONOUS EOSINOPHILIC SINUS AND NASAL INFLAMMATION WAS DEVELOPED BY SENSITIZING BALB/c MICE TO ASPERGILLUS FUMIGATUS EXTRACTS. THIS STUDY SOUGHT TO DEVELOP THIS MURINE MODEL FURTHER INTO A MODEL OF CHRONICALLY INFAMED SINONASAL MUCOSAL WOUND HEALING AND ESTABLISH THE FEASIBILITY OF EVALUATING DIFFERENCES IN WOUND HEALING RESPONSES IN NORMAL AND CHRONICALLY INFAMED SINUS MUCOSA.

METHODS

INDUCING CHRONIC EOSINOPHILIC INFLAMMATION

The methods of Lindsay et al in inducing chronic eosinophilic nasal and sinus inflammation in female BALB/c mice (7-8 weeks of age, Taconic Farms, Germantown, New York) were duplicated in our study. A mixture of culture filtrate and mycelial extracts of A fumigatus (Hollister-
underwent histologic and gene expression analysis. Male and female mice were killed before and 3, 7, and 14 days after wounding and injury created by endoscopic sinus surgery. Mice were housed any external trauma and more accurately mimicked the anterior to create direct mucosal injury within the maxillary sinus. The sinus was at the external naris. It was pulled from posterior to anterior to create direct mucosal injury within the maxillary sinus. A Rosen needle was placed through the nostril until the mark was established via use of a Rosen needle, previously marked along the slitlike maxillary ostium laterally. Mice have the right nasal and sinus mucosa were harvested as 1 block and the timeline in the histologic analysis. The histologic characteristics of secretory hyperplasia are increased goblet cell concentration and the prominence of mucus overlying surface epithelium.

**Figure 1.** A flow diagram shows the groups of mice involved and the timeline in the histologic analysis. A.f. indicates Aspergillus fumigatus.

**Figure 2.** A flow diagram shows the groups of mice involved and the timeline in the analysis of gene expression.

Stier Laboratories, Spokane, Washington) was used for intra-peritoneal and 3-times weekly intranasal use for 10 weeks. The study protocol was reviewed and approved by the University of Utah Institutional Animal Care and Use Committee (IACUC).

Once eosinophilic inflammation was confirmed by light microscopy, the animals underwent wounding of the sinonasal cavity. Following anesthesia via inhalation of isoflurane, wounding was established via use of a Rosen needle, previously marked to 6.2 mm from the tip as determined from prior work on female BALB/c mice. This measurement placed the tip of the needle along the slitlike maxillary recess ostium laterally. Mice have a slitlike maxillary ostium running nearly the entire distance of the recess, communicating widely into the nasal cavity. The Rosen needle was placed through the nostril until the marking was at the external naris. It was pulled from posterior to anterior to create direct mucosal injury within the maxillary recess, lateral nasal wall, and nasal floor. The technique bypassed any external trauma and more accurately mimicked the injury created by endoscopic sinus surgery. Mice were humanely killed before and 3, 7, and 14 days after wounding and underwent histologic and gene expression analysis.

**HISTOLOGIC EXAMINATION**

For the histologic analysis, each time point group had 7 animals. Seven mice that had not been exposed to A. fumigatus extract served as uninfamed, nonwounded controls. After lethal injection, the mandible was removed and the head was placed in 10% neutral-buffered formalin for 24 hours for initial fixation and then decalified. The skulls were then sectioned coronally into the following 3 regions for paraffin embedding: (A) caudal to the incisor teeth, (B) halfway between A and C, and (C) anterior margin of the orbit. Sections were approximately 2 to 3 mm thick and slides were prepared 0.5-µm thick from the face of each section. Hematoxylin-eosin (HE) was used to characterize the inflammatory infiltrate at standardized anatomic locations according to nasal diagrams. The presence and degree of inflammation were assayed by light microscopic examination by a blinded veterinary pathologist. The sections were scored for inflammation, secretory hyperplasia, edema, fibrosis, and epithelialization using a 5-point scale: 0, none; 1, minimal; 2, mild; 3, moderate; and 4, severe/complete. **Figure 1** is a flow diagram showing the groups involved and the timeline in completing the histologic analysis. The histologic characteristics of secretory hyperplasia are increased goblet cell concentration and the prominence of mucus overlying surface epithelium.

**GENE EXPRESSION ANALYSIS**

For the gene expression analysis, inflamed mice as well as uninfamed mice were wounded and subsequently killed, with each time point group having 4 animals. One group of 4 inflamed mice and 1 group of 4 uninflamed mice served as nonwounded controls. **Figure 2** is a flow diagram showing the groups involved and the timeline in completing the polymerase chain reaction (PCR) analysis.

Following lethal injection the sinuses were exposed, and the right nasal and sinus mucosa were harvested as 1 block and placed into RNAlater solution (Quiagen, Valencia, California) to protect from RNA degradation and stored at −70°C until analyzed.

Total messenger RNA (mRNA) isolation was performed as previously described. The quantity of the RNA was determined by absorbance at 260 nm, and the quality examined by gel electrophoresis. Complementary DNA (cDNA) was prepared using the First Strand cDNA Synthesis Kit (Roche Applied Science, Indianapolis, Indiana) in a volume of 20 µL, containing 2 µg of DNase 1-treated RNA, 20mM deoxynucleotide triphosphate (dNTP) mix, 1.6 µg of Oligo-p(dT)15 primer, 50 U of RNase inhibitor, 5mM magnesium chloride, and 25 U of avian myeloblastosis (AMV) reverse transcriptase. The reaction was carried out at 42.0°C for 95 minutes, then, brought to 100 µL with ribonuclease (RNase)-free water. Primers for interleukin 1B (IL-1B), transforming growth factor (TGF)-β1, tumor necrosis factor (TNF), insulinlike growth factor 1 (IGF-1), matrix metalloproteinase (MMP)-7, MMP-9, Tissue inhibitor of mettalloproteinase 1 (TIMP-1), and prostataglandin E receptor EP4 (PTGER-4) were used in this study (Table 1). The primers used in this study were designed using the Primer 3 program (Whitehead Institute for Biomedical Research/MIT Center, Cambridge, Massachusetts), based on the sequences obtained from the GenBank database. The correct sequences of the PCR products were verified by DNA sequencing. The optimized conditions were adapted to the LightCycler PCR protocol. Real-time PCR was performed on a LightCycler (Roche Applied Science), with conditions optimized previously. Tests were performed in duplicate, and the mean copy number was calculated for each gene, relative to Gapdh (GenBank M32599) (glyceraldehyde-3-phosphate dehydrogenase) expression. This method involved quantifying the housekeeping gene.
Gapdh in the samples and then normalizing samples by physical dilution based on Gapdh concentration. This method had previously been performed and described in work comparing differential gene expression in tissue samples with allergic fungal sinusitis and eosinophilic mucin rhinosinusitis.10

STATISTICAL ANALYSIS

Means and standard deviations of the ordinal composite scores for each histological determination (inflammation, secretory hyperplasia, edema, fibrosis, and epithelialization) were calculated. There was insufficient variation to analyze the data using a Mann-Whitney test.

Gene expressions were analyzed with a 2-tailed unpaired t test (GraphPad Prism 5.00; GraphPad Software, San Diego, California). P < .05 was considered significant. Despite multiple t tests performed, a Bonferroni correction was not used in this exploratory pilot study.

RESULTS

Wounding was reliably performed using a Rosen needle to injure along the entire length of the maxillary recess ostium. Figure 3 depicts a gross section of the area of wounding at the maxillary sinus recess ostium. The nostril is oriented to the right and the brain to the left.

Figure 4. Hematoxylin-eosin–stained coronal cut through the mouse head at an original magnification of ×4. A, The left nasal cavity; and B, the right nasal cavity from the same slide (an arrow points to the injury at the maxillary sinus recess ostium with blood noted in the ostium and thinning of the normal pseudostratified columnar epithelium found on the uninjured side in panel A).

INFLAMMATORY MEDIATOR EXPRESSION PATTERNS

Table 2 depicts the expression patterns of the 8 inflammatory genes in the uninflamed and inflamed mice. There was no significant difference seen in Gapdh expression between uninflamed and inflamed groups. We found inter-
In this project the methods of Lindsay et al were replicated and verified via histologic analysis to establish that a reliable murine model of chronic rhinosinusitis could be achieved in our laboratory. We then attempted to mimic endoscopic sinus surgery by introducing a Rosen needle through the nostril and using this to injure the sinonasal mucosa. Confirmation of appropriate wounding was achieved by histopathologic examination of wounded experimental mice. This technique required limited anesthesia time, roughly 30 seconds, which was easily achieved with the inhalational agent isoflurane, and no mortality events were encountered using this wounding protocol. We demonstrated increased inflammation at baseline (unwounded) and after wounding in mice exposed to *A fumigatus*. For many of the genes tested, this gap appeared to narrow over the course of the experiments. We did not continue the *A fumigatus* extract after wounding, which may account for the closure of the inflammatory gap for many of the genes studied.

### Table 2. Expression Patterns of the 8 Inflammatory Mediator Genes as Depicted by the Mean Relative Concentrations (Averaged Over 4 Mice) Found for Each Gene.

<table>
<thead>
<tr>
<th>Gene Name</th>
<th>0 (Nonwounded)</th>
<th>3</th>
<th>7</th>
<th>14</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>TGF-β1β</strong></td>
<td></td>
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</tr>
<tr>
<td>No <em>A fumigatus</em></td>
<td>4.1 (1.0)</td>
<td>8.5 (4.0)</td>
<td>18.3 (6.7)</td>
<td>97.7 (50.7)</td>
</tr>
<tr>
<td><em>A fumigatus</em></td>
<td>9.0 (1.9)</td>
<td>12.0 (4.5)</td>
<td>18.5 (1.1)</td>
<td>54.4 (16.6)</td>
</tr>
<tr>
<td><strong>IL-1β</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>No <em>A fumigatus</em></td>
<td>8.4 (3.1)</td>
<td>47.2 (13.6)</td>
<td>6.2 (2.1)</td>
<td>9.0 (9.2)</td>
</tr>
<tr>
<td><em>A fumigatus</em></td>
<td>89.8 (89.8)</td>
<td>30.1 (6.9)</td>
<td>9.3 (2.9)</td>
<td>20.3 (9.2)</td>
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<tr>
<td><strong>TNF</strong></td>
<td></td>
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<tr>
<td>No <em>A fumigatus</em></td>
<td>0.3 (0.1)</td>
<td>4.0 (1.5)</td>
<td>0.5 (0.3)</td>
<td>4.7 (3.1)</td>
</tr>
<tr>
<td><em>A fumigatus</em></td>
<td>5.3 (4.3)</td>
<td>4.3 (1.2)</td>
<td>0.8 (0.2)</td>
<td>4.5 (1.7)</td>
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<tr>
<td><strong>IGF-1</strong></td>
<td></td>
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<tr>
<td>No <em>A fumigatus</em></td>
<td>1.3 (0.7)</td>
<td>1.6 (0.7)</td>
<td>1.6 (0.7)</td>
<td>4.6 (2.0)</td>
</tr>
<tr>
<td><em>A fumigatus</em></td>
<td>9.0 (2.0)</td>
<td>6.9 (2.0)</td>
<td>2.7 (1.9)</td>
<td>3.1 (0.4)</td>
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<tr>
<td><strong>MMP-7</strong></td>
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<tr>
<td>No <em>A fumigatus</em></td>
<td>0.1 (0.0)</td>
<td>0.1 (0.0)</td>
<td>0.2 (0.1)</td>
<td>0.6 (0.3)</td>
</tr>
<tr>
<td><em>A fumigatus</em></td>
<td>0.3 (0.3)</td>
<td>0.3 (0.2)</td>
<td>0.3 (0.0)</td>
<td>0.8 (0.6)</td>
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<tr>
<td><strong>MMP-9</strong></td>
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<tr>
<td>No <em>A fumigatus</em></td>
<td>18.1 (7.5)</td>
<td>8.3 (2.9)</td>
<td>26.6 (11.8)</td>
<td>51.3 (36.6)</td>
</tr>
<tr>
<td><em>A fumigatus</em></td>
<td>7.8 (2.7)</td>
<td>6.2 (3.9)</td>
<td>9.1 (3.7)</td>
<td>37.1 (18.3)</td>
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<tr>
<td><strong>TIMP-1</strong></td>
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<td></td>
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<tr>
<td>No <em>A fumigatus</em></td>
<td>14.8 (5.1)</td>
<td>89.2 (7.5)</td>
<td>42.4 (22.4)</td>
<td>42.2 (28.8)</td>
</tr>
<tr>
<td><em>A fumigatus</em></td>
<td>33.3 (8.4)</td>
<td>130.1 (12.3)</td>
<td>26.1 (4.6)</td>
<td>49.8 (18.2)</td>
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<tr>
<td><strong>PTGER-4</strong></td>
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<td></td>
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<tr>
<td>No <em>A fumigatus</em></td>
<td>0.7 (0.2)</td>
<td>1.3 (0.5)</td>
<td>1.2 (0.6)</td>
<td>3.7 (0.7)</td>
</tr>
<tr>
<td><em>A fumigatus</em></td>
<td>2.1 (0.5)</td>
<td>1.3 (0.9)</td>
<td>1.4 (0.3)</td>
<td>3.1 (1.2)</td>
</tr>
</tbody>
</table>

**Abbreviations:** IL-1β, interleukin 1β; IGF-1, insulin-like growth factor 1; MMP, matrix metalloproteinase; TGF-β1, transforming growth factor β1; TNF, tumor necrosis factor; TIMP-1, tissue inhibitor of metalloproteinase 1; PTGER-4, prostaglandin E receptor EP4.

*Data are expressed in thousands (ie, 4.1=4100) with standard deviations in parentheses. Relative concentration was calculated by multiplying the average protein level by the Gapdh (glyceraldehyde 3-phosphate dehydrogenase) index.*

**COMMENT**

Despite the frequency of sinus surgery, relatively little is known about wound healing in the paranasal sinuses. Data from skin wounding studies are not likely applicable owing to the difference in epithelium, local environment, and degree of inflammation prior to wounding. Data from laryngeal wounding studies have shown changes in certain cytokines and inflammatory markers that shed some light on airway mucosal healing, but these studies involved partial-thickness, whereas sinus surgery involves full-thickness wounds, often with exposed underlying bone.1,12

1. Lindsay et al.

In this project the methods of Lindsay et al were replicated and verified via histologic analysis to establish that...
Aspergillus fumigatus resulted in statistically significant differences in gene expression for IGF-1, TIMP-1, and MMP-9 prior to wounding and continued after wounding during the healing process. These cytokines have shown changes during wound healing in nonsinus mucosal tissues. Interestingly, MMP-9 levels were decreased in inflamed animals compared with uninflamed animals. Matrix metalloprotease 9 expression has been shown to be increased in chronic rhinosinusitis, with its degree of expression correlating with the quality of mucosal healing following sinus surgery. Kostamo et al found lower levels of MMP-9 to be associated with eosinophilic vs noneosinophilic inflammation, while Watelet et al found that lower levels of MMP-9 at baseline cor-

Figure 6. Comparison of copy numbers per microliter of MMP-9 (A), TIMP-1 (B), TGF-β1b (C), and IGF-1 (D) in wounded normal and inflamed mice.

‡, Significant difference in chronic sinonasal inflamed mucosa compared with normal mucosa; †, Significant change due to wounding in normal mucosa when compared with nonwounded controls; *, Significant change due to wounding in inflamed mucosa when compared with nonwounded inflamed controls. Day 0 depicts nonwounded controls. No Af indicates Aspergillus fumigatus; IGF-1, insulin-like growth factor 1; MMP-9, matrix metalloproteinase 9; TIMP-1, tissue inhibitor of metalloproteinase 1; and TGF-β1b, transforming growth factor β1b.
related with better postoperative wound healing. Determining any clinical significance of the MMP-9 data in our small homogenous sample of mice may be premature, yet these data substantiate the potential importance of this marker in sinus wound healing.

Tissue inhibitor of metalloproteinase 1 and MMP-9 are important proteins involved in remodeling of the extracellular matrix during wound healing. Levels of TIMP-1 peaked 3 days after wounding and were significantly higher in inflamed mucosa. This temporal expression pattern did not correlate with the MMP-9 expression but does suggest that TIMP-1 is involved early on in regulating wound remodeling in both inflamed and uninflamed mucosa.

Limitations of this study can be attributed to the small sample size for each time point in this initial study. Also, a P value of <.05 was used despite the number of data points examined. Despite the less stringent P value, the data from this pilot study provide insight into markers for future testing on human sinonasal tissue. Additional studies using larger sample sizes will be necessary to confirm our PCR findings. Moreover, evaluation was limited to 2 weeks following wounding. Using additional time points to follow the progression of inflammation would provide us with a better understanding of longer-term wound healing especially in chronically inflamed tissue. The postsurgical practice of irrigation and debridement (and its inherent rewounding of the mucosa) could also be assessed. Future direction will focus on validation of this model by comparing with human sinonasal wounds.

In conclusion, we successfully constructed an animal model in which wounding in a setting of chronic eosinophilic inflammation can be studied. We further found the chronic eosinophilic inflammation generated by A fumigatus instillation appears to diminish rapidly without continuation of the A fumigatus instillation. The histologic and PCR results confirmed the utility of this model and its potential use in examining the effect of steroids, biomaterials, and other wound healing and inflammatory modifiers. The number of mice used in this exploratory pilot study to look at mRNA expression was small; however, there appeared to be possible differences in the inflammatory response after wounding in IGF-1, TIMP-1, and MMP-9. More research with larger sample sizes will need to be conducted to better investigate the impact of chronic inflammation on the wound healing process. The model developed herein, however, appears to be well suited for this line of research.

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Author Contributions: Dr Tansavatdi had full access to all the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis. Study concept and design: Tansavatdi and Orlandi. Acquisition of data: Tansavatdi, McGill, Riggs, and Orlandi. Analysis and interpretation of data: Tansavatdi and Orlandi. Drafting of the manuscript: Tansavatdi, McGill, and Riggs. Critical revision of the manuscript for important intellectual content: Tansavatdi and Orlandi. Statistical analysis: Orlandi. Obtained funding: Orlandi. Administrative, technical, and material support: Tansavatdi, McGill, Riggs, and Orlandi. Study supervision: Tansavatdi and Orlandi.

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Additional Contributions: Abdel-Aziz El Sherif, MD, contributed with data acquisition and D. Glen Esplin, DVM, PhD, contributed to the histologic analysis of the samples for this project.

REFERENCES