

Altered expression of tissue remodeling genes in a mouse model of acute allergic rhinitis

Nathan B. Sautter, MD¹, Katherine L. Delaney, BS², Dennis R. Trune, PhD²

Background: Osteogenesis, fibrosis, and scarring are prominent pathologic changes resulting from chronic sinonasal inflammation, and these tissue changes may increase the degree of disease symptomatology and the level of surgical difficulty. Members of the bone morphogenetic protein (BMP) and fibroblast growth factor (FGF) families of cytokines and the matrix metalloproteinase (MMP) family of endopeptidases are known to regulate tissue remodeling in other disease processes, but their role in acute and chronic sinonasal inflammation remains undefined.

Methods: A previously described mouse model of acute allergic rhinitis secondary to *Aspergillus fumigatis* exposure in BALB/C mice was used. Intranasal challenge was performed 1 week following intraperitoneal sensitization with *A. fumigatis* extract and mice were sacrificed 6 hours ($n = 8$) and 24 hours ($n = 8$) later. Additional mice were intranasally challenged 3 times per week and sacrificed at the end of 7 days ($n = 8$) and 21 days ($n = 8$). The snouts were processed for quantitative reverse-transcription polymerase chain reaction (RT-PCR) and compared to untreated controls for messenger ribonucleic acid (mRNA) expression of BMP1, 2, 3, 4, 5, 6, 7, 8a, 8b, 9, 10, FGF1, 2, 3, 4, 5, 6, 7, 8, 10, and MMP1a, 2, 3, 7, 8, 9, 12, and 14. Additional 21-day-old mice were prepared for sinonasal histopathology. Control mice were treated with the same protocol, with intraperitoneal phosphate-buffered saline (PBS) and intranasal PBS substituted for *A.*

fumigatis extract. Untreated mice were used for additional comparison.

Results: Compared to both the PBS-treated and untreated control groups, statistically significant ($p < 0.05$) upregulation of MMP8 was observed in the 6-hour time point. Significant downregulation of MMP8 was observed at 1 week. Significant upregulation of FGF3 was observed at 1 week ($p < 0.05$). BMP3 and BMP5 were significantly downregulated in the 1-week group ($p < 0.05$). The mice exhibited histologic sinonasal changes consistent with allergic inflammation.

Conclusion: Intranasal exposure to *A. fumigatis* results in altered expression of several tissue remodeling cytokines at varying time points in the acute allergic rhinitis mouse model. These changes in cytokine regulation may subsequently contribute to sinonasal osteogenesis, scarring, and fibrosis as seen in chronic rhinosinusitis. © 2011 ARS-AAOA, LLC.

Key Words: allergic rhinitis; BMP; FGF; MMP; mouse model; tissue remodeling

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¹Department of Otolaryngology-Head and Neck Surgery, Oregon Health Sciences University, Portland, OR; ²Oregon Hearing Research Center, Oregon Health Sciences University, Portland, OR

Correspondence to: Nathan B. Sautter, MD, Assistant Professor, Dept. of Otolaryngology-Head and Neck Surgery, Oregon Health Sciences University, 3181 SW Sam Jackson Park Road, PV-01, Portland, OR 97239; e-mail: sauttern@ohsu.edu

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It is widely believed that chronic rhinosinusitis (CRS) is a spectrum of multifactorial diseases sharing a common factor of chronic sinonasal mucosal inflammation. Research has demonstrated that dysfunction of cytokine expression plays a key role in CRS and allergic inflammation.^{1,2} Tissue remodeling as a result of sinonasal osteogenesis, fibrosis, angiogenesis, and scarring is a common finding in patients with longstanding CRS.³⁻⁵ In addition, proliferation of the extracellular matrix is seen in CRS with nasal polypsis.⁶ These changes affect normal sinonasal physiology and increase disease symptomatology, as well as surgical difficulty. Shared clinical experience suggests that persistent localized inflammation in patients

with sinonasal osteogenesis and osteitis may be resolved only through removal of the underlying bone.⁵ Some authors have investigated the role of osteitis and osteogenesis in CRS, but the underlying molecular mechanisms that ultimately lead to tissue remodeling in CRS remain largely uncharacterized.^{3,4,7,8}

Members of the bone morphogenetic protein (BMP) and fibroblast growth factor (FGF) families of cytokines are responsible for regulation of tissue growth and remodeling in many normal physiologic processes, such as embryological development, bone growth, wound healing, angiogenesis, and regulation of inflammation. Matrix metalloproteinases (MMPs) act as proteolytic enzymes that regulate remodeling of the extracellular matrix, as well as cytokine activity.^{9,10} MMP dysregulation plays a role in many pathologic processes, including cancer, atherosclerosis, neurodegenerative diseases, and cholesteatoma, as well as inflammatory diseases such as otitis media, CRS, and asthma.^{11–21} BMP dysregulation is seen in gastric and colon malignancies, and FGF signaling is known to play a key role in many types of cancer.^{9,22,23} Unlike the MMP family, however, little is known regarding the role of BMP and FGF family members in CRS.^{24,25}

Tissue remodeling is routinely observed in animal models of CRS. The murine model of CRS, initially described by Lindsay et al.,²⁶ exhibits many of the same histologic changes seen in human CRS, including thickening of the lamina propria, mucosal inflammatory cell infiltrate, and increase in nonciliated epithelial cells with increased apocrine secretion.^{27,28} Bony remodeling of the sinonasal cavity, including osteogenesis, osteitis, and periosteal fibrosis, has been described in several studies using a rabbit model of bacterial CRS.^{7,29–31}

The underlying molecular mechanisms governing CRS-associated tissue remodeling, however, remain uncharacterized in animal models as well as in human subjects with CRS. A better understanding of the cellular and molecular processes leading to osteogenesis, fibrosis, and angiogenesis in CRS is needed in order to develop novel therapies aimed at halting or reversing CRS-associated tissue remodeling. The purpose of this study is to characterize the initial changes in BMP, FGF, and MMP family expression in a mouse model of acute allergic rhinitis. We hypothesize that induction of rhinitis following fungal antigen exposure will result in changes in the level of regulation of several members of the BMP, FGF, and MMP families.

Subjects and methods

Acute allergic rhinitis mouse model

The murine model as described by Lindsay et al.²⁶ and later modified by Khalid et al.²⁷ was employed. BALB/C mice 8 to 12 weeks of age were presented with an intraperitoneal challenge of 200 μ g of *Aspergillus fumigatus* (AF) extract (Greer Laboratories, Inc., Lenoir, NC) dissolved in 2 mg of alum and 0.5 mL of phosphate buffered saline (PBS).

Beginning 7 days following intraperitoneal sensitization, bilateral intranasal challenge with 5 μ g of AF extract was administered under anesthesia using a subcutaneous injection of ketamine (100 mg/mL; 0.067 mg/gm body weight) and xylazine (20 mg/mL; 0.013 mg/gm body weight). Mice were sacrificed at 6 hours ($n = 8$) and 24 hours ($n = 8$) following a single intranasal challenge. Additional mice were challenged 3 times per week until sacrificed at 7 days ($n = 8$) and 21 days ($n = 8$) following the initial nasal challenge, resulting in a total of 3 and 9 intranasal treatments, respectively. A final group of mice was treated with intraperitoneal and intranasal PBS instead of AF extract, and snouts were harvested at the time of sacrifice after 1 week of treatment ($n = 8$). Completely untreated mouse snouts ($n = 8$) were used as controls. At the time of sacrifice, the sinonasal mucosa and the underlying vomeronasal and ethmoturbinate bone were promptly dissected from the snouts and placed immediately in RNAlater solution (Qiagen, Valencia, CA).

Quantitative real-time reverse-transcriptase polymerase chain reaction

RNA for real-time reverse-transcriptase polymerase chain reaction (RT-PCR) was extracted from dissected sinonasal tissue using the RNeasy Mini Kit according to the manufacturer's instructions (Qiagen). A total of 200 μ g of RNA was used to synthesize the complementary DNA (cDNA) probe using the C-03 RT² first strand kit (SABiosciences, Frederick, MD). Standardized custom PCR kits optimized for each cytokine, including the primer and probe, were utilized (SABiosciences). Custom arrays were obtained for the following cytokines: BMP1, BMP2, BMP3, BMP4, BMP5, BMP6, BMP7, BMP8a, BMP8b, BMP9, BMP10, FGF1, FGF2, FGF3, FGF4, FGF5, FGF6, FGF7, FGF8, FGF10, MMP1a, MMP2, MMP3, MMP7, MMP8, MMP9, MMP12, and MMP14. The method of semiquantitative real-time RT-PCR was utilized, and reverse transcription was performed using an ABI Step One Plus system (Applied Biosystems, Foster City, CA). Using this system, the parameter C_t (threshold cycle) is defined as the fractional cycle number at which the reporter fluorescence generated by cleavage of the probe passes a fixed threshold above baseline. A plot of the log of initial target copy number for a set of standards vs C_t is a straight line. Quantitation of the amount of target in samples is done by measuring C_t and using the standard curve to determine starting copy number. Calculation of C_t s, preparation of a standard curve, determination of the starting copy number, and calculation of statistical significance was performed using the $\Delta\Delta C_t$ method with the aid of the SABiosciences PCR Array Data Analysis Web Portal. 18S RNA was used as the comparator.

Calculation of $\Delta\Delta C_t$ and statistical analysis

Due to the inverse proportional relationship between the C_t and the original gene expression level, and the doubling of

the amount of product with every cycle, the original expression level (L) for each gene of interest (GOI) is expressed as:

$$L = 2^{-C_t} \quad [1]$$

To normalize the expression level of a GOI to a house-keeping gene (HKG), the expression levels of the 2 genes are divided:

$$\frac{2^{-C_t(\text{GOI})}}{2^{-C_t(\text{HKG})}} = 2^{-[C_t(\text{GOI}) - C_t(\text{HKG})]} = 2^{-\Delta C_t} \quad [2]$$

To determine fold change in gene expression, the normalized expression of the GOI in the experimental sample is divided by the normalized expression of the same GOI in the control sample:

$$\frac{2^{-\Delta C_t(\text{expt})}}{2^{-\Delta C_t(\text{control})}} = 2^{-\Delta \Delta C_t}$$

$$\text{Where } \Delta \Delta C_t \text{ is equal to } \Delta C_t(\text{expt}) - \Delta C_t(\text{control}) \quad [3]$$

The complete calculation is as follows:

$$\begin{aligned} \frac{\frac{2^{-\Delta C_t(\text{GOI})_{\text{expt}}}}{2^{-\Delta C_t(\text{HKG})_{\text{expt}}}}}{\frac{2^{-\Delta C_t(\text{GOI})_{\text{control}}}}{2^{-\Delta C_t(\text{HKG})_{\text{control}}}}} &= \frac{2^{-[C_t(\text{GOI}) - C_t(\text{HKG})]_{\text{expt}}}}{2^{-[C_t(\text{GOI}) - C_t(\text{HKG})]_{\text{control}}}} \\ &= \frac{2^{-\Delta C_t_{\text{expt}}}}{2^{-\Delta C_t_{\text{control}}}} = 2^{-\Delta \Delta C_t} \end{aligned} \quad [4]$$

In order to determine statistical significance, a 2-tailed *t* test assuming equal variance was performed using the $\Delta \Delta C_t$ values as input.

Histologic analysis

Experimental mice were treated according to the above protocol using AF extract. Mice were sacrificed after 3 weeks of nasal challenge. The snouts were harvested, fixed in 3% paraformaldehyde, embedded in paraffin, sectioned at 5 μm , and stained. Tissues were observed with light microscopy for histopathologic changes. Serial sections from 2 treated mice snouts were analyzed, and untreated mouse snouts were used as controls for comparison.

The histopathologic grading scale as described by Khalid et al.²⁷ was used. Every fifth section containing the vomeronasal organ and nasal-associated lymphoid tissue was examined and graded. Inflammation was graded from 0 (no change) to 4 (severe change), and secretory hyperplasia was also graded from 0 (no change) to 4 (severe change). Control sections were used to define the grade of 0. The histologic grades of the treated snouts were then averaged and compared to the untreated controls.

All animal procedures were approved by the Oregon Health Sciences University (OHSU) Institutional Animal Care and Use Committee.

Results

Messenger RNA expression

Initial comparison of the untreated control group and the PBS-only treatment group revealed that a mild degree of change in messenger RNA (mRNA) expression was elicited by the PBS-only treatment. In many cases, the changes in mRNA expression following PBS-only treatment paralleled the changes observed in the AF treatment groups, and no statistically significant difference was observed between the 2 groups. In select cases, the degree of change in gene expression in the AF treatment group exceeded that seen in the PBS-only treatment group. Therefore, we considered a change in gene expression significant only if it demonstrated a statistically significant difference compared to the level of expression in both the untreated control group and the PBS-only treatment group.

In the MMP family, only MMP8 demonstrated a significant change in expression compared to the untreated control and the PBS-only treatment group (Fig. 1). Compared to the PBS-treated controls, MMP8 was upregulated at 6 hours by a factor of 25.3 ($p = 0.008$; 95% confidence interval [CI], 6.4–46.1), and downregulated at 1 week by 41% ($p = 0.04$; 95% CI, 0.22–0.96). A trend in MMP8 upregulation by a factor of 1.8 was noted at 24 hours, but this did not reach statistical significance. No significant expression of MMP1a, MMP7, and MMP12 was detected in the control or treatment groups. MMP2, MMP3, MMP9, and MMP14 were all expressed in the sinonasal tissue, but no significant change in expression was observed following AF treatment.

Analysis of FGF family expression revealed a significant change in FGF3 expression (Fig. 2). Compared to the PBS-treated controls, FGF3 was upregulated by a factor of 9.5 at 1 week ($p = 0.0006$, 95% CI, 5.2–13.8). FGF3 exhibited an upward trend in regulation at 6 hours (factor of 2.2), 24 hours (factor of 1.3), and 3 weeks (factor of 1.4), although none of these reached statistical significance. No significant expression of FGF4, FGF5, FGF6, and FGF8 was noted in the control or treatment groups. FGF1, FGF2, FGF7, and FGF10 were all expressed in the sinonasal tissue, but no significant change in expression was observed following AF treatment.

Significant downregulation was noted in both BMP3 and BMP5 (Fig. 3). Compared to the PBS-treated controls, BMP3 was downregulated by 38% at 1 week ($p < 0.01$; 95% CI, 0.41–0.83), and BMP5 was downregulated by 25% at 1 week ($p < 0.02$; 95% CI, 0.53–0.98). No significant expression of BMP8a or BMP10 was noted in either the control or treatment groups. BMP1, BMP4, BMP5, BMP6, BMP7, and BMP8b were all expressed in the sinonasal tissue, but no significant change in expression was observed following AF treatment.

Histologic analysis

Histologic analysis of the AF-treated mouse snouts revealed hypertrophy of the underlying stroma and basal lamina

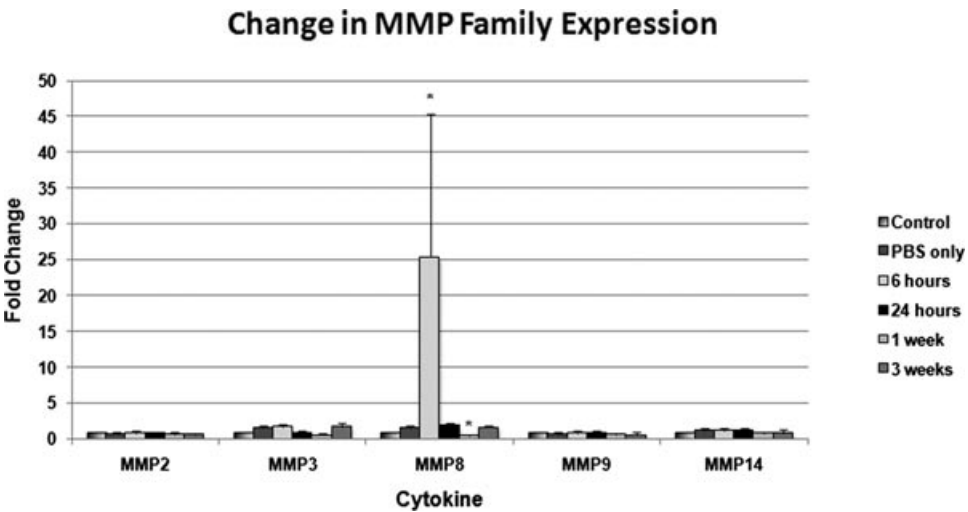


FIGURE 1. Change in regulation of members of the MMP family over time. Statistically significant time points as compared to both the control and PBS-only groups are labeled with an asterisk (*). Error bars are included to indicate 95% confidence intervals.

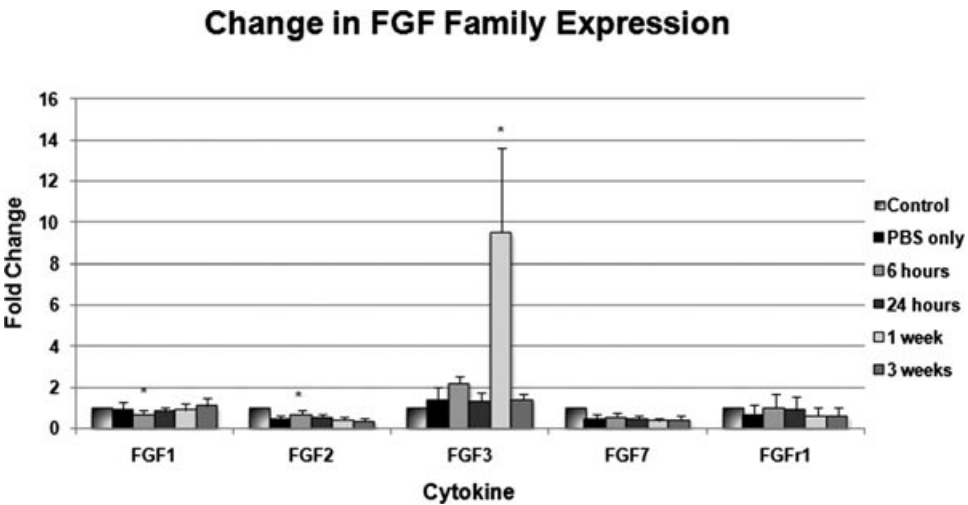


FIGURE 2. Change in regulation of members of the FGF family over time. Statistically significant time points as compared to both the control and PBS-only groups are labeled with an asterisk (*). Error bars are included to indicate 95% confidence intervals.

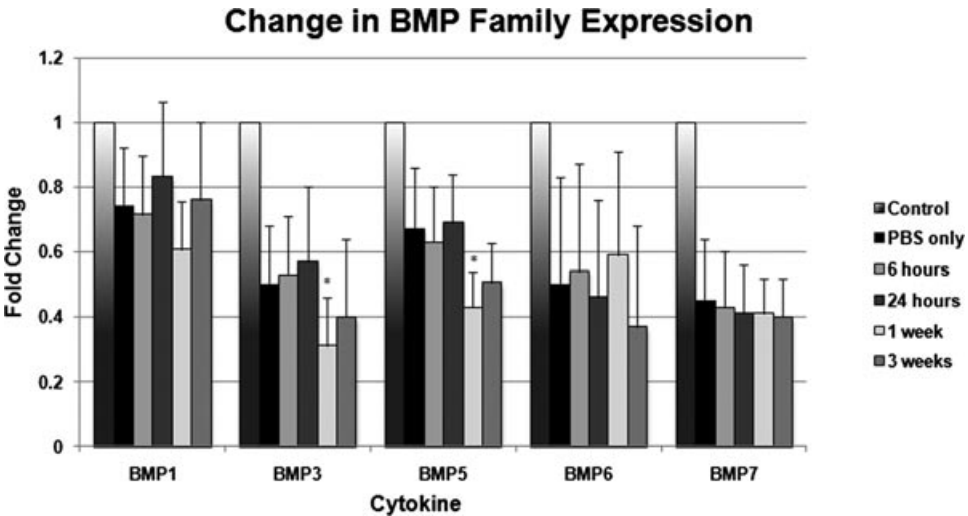


FIGURE 3. Change in expression of members of the BMP family over time. Statistically significant time points as compared to both the control and PBS-only groups are labeled with an asterisk (*). Error bars are included to indicate 95% confidence intervals.

with submucosal inflammatory cell infiltrate (Fig. 4B), increased apocrine secretion with apical blebbing, mucous gland hypertrophy, and loss of cilia (Fig. 4C,D) compared to untreated controls (Fig. 4A). According to the Khalid grading scale, the average inflammatory change was 1.7 and the average change in secretory hyperplasia was 1.4. No obvious bony changes were noted.

Discussion

These studies show significant regulatory changes in various tissue remodeling cytokines and enzymes over time in the mouse model of acute allergic rhinitis. To our knowledge, this is the first study to investigate tissue remodeling cytokine expression in an allergic mouse model. We observed both upregulation and downregulation in several members of the BMP, FGF, and MMP families in a time-dependent manner. In particular, MMP8 was upregulated by a factor of 25, FGF3 was upregulated by a factor of 7, and BMP3 and BMP5 were both significantly downregulated.

Little is known about the role of cytokines and MMPs in tissue remodeling in chronic inflammatory diseases. Although members of the BMP family have been hypothesized to play a role in sinonasal osteogenesis and osteitis in CRS, no studies have formally investigated their association with sinonasal inflammation.²⁴ Only 1 other study has described a role for FGF cytokines in CRS. Ishibashi et al.²⁵ demonstrated upregulation of keratinocyte growth factor (FGF7) in nasal polyps. In the current study, upregulation and downregulation of various members of the MMP, FGF, and BMP families occurs in a time-dependent manner in the mouse model. This suggests that molecular signaling for bony remodeling and fibrosis is a dynamic process that is initiated early in inflammatory disease.

Several authors have investigated the role of MMPs in CRS. MMP2, MMP7, and MMP9 upregulation in nasal polyposis has been demonstrated in multiple prior studies.^{16,18,20,21} Although we did not observe any such changes in the present study, it is possible that changes in MMP2, MMP7, and MMP9 expression may be seen at time points later than 3 weeks. A significant finding in our study is the notable increase of MMP8 at the 6-hour time point. MMP8 is a proinflammatory factor that acts as a promoter of neutrophil chemotaxis. Additionally, it is an interstitial collagenase that promotes collagen breakdown.¹⁹ The significant upregulation at 6 hours suggests MMP8 may play a role in both initiation of sinonasal inflammation as well as breakdown of the extracellular matrix in the mouse model of allergic rhinitis.

FGF3 exhibited significant upregulation at 1 week, with trends toward significance at other time points. FGF3 is known to play an important role in neural and inner ear development; sensorineural hearing loss, microtia, and microdontia have been reported as a consequence of FGF3 mutation.³² To our knowledge, the present study is the first to suggest a role for FGF3 in sinonasal disease.

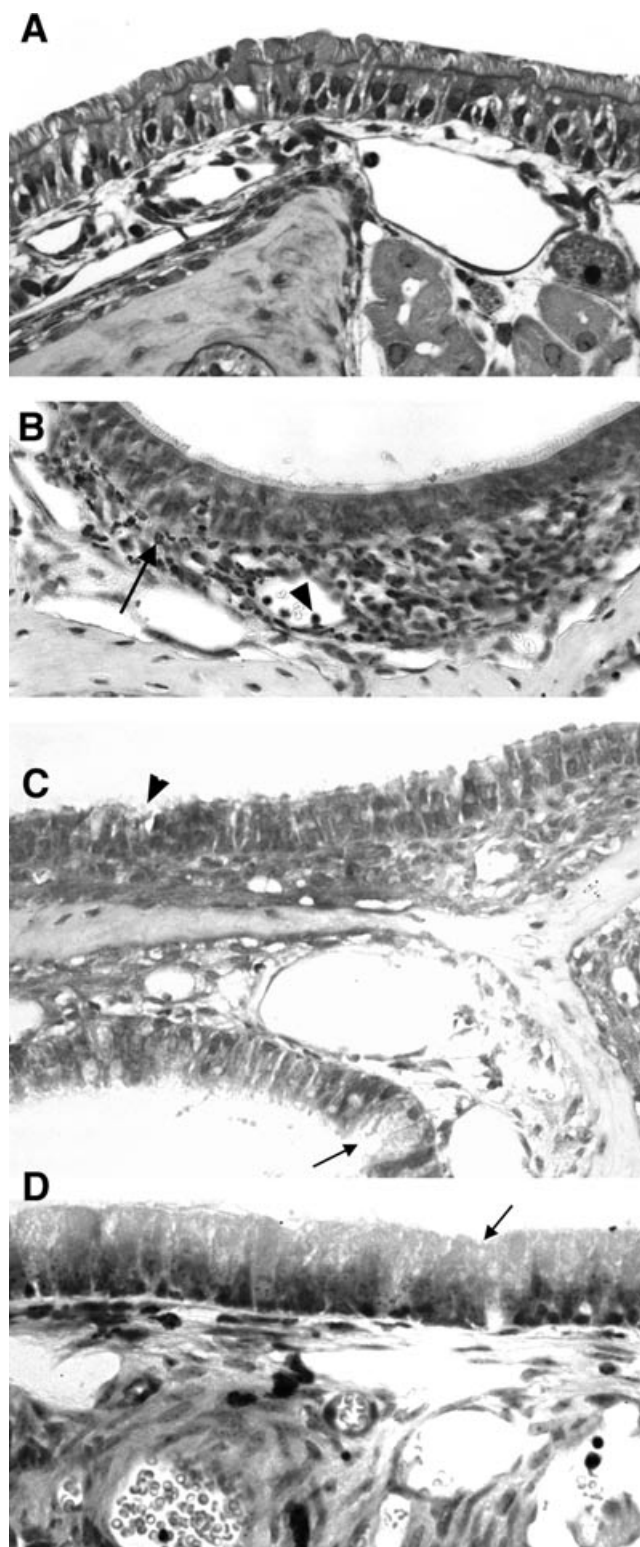


FIGURE 4. Micrographs ($\times 40$) of (A) untreated and (B-D) *Aspergillus*-treated mouse ethmoturbinates. (B) Thickening of the underlying stroma with inflammatory cell infiltrate including eosinophils (arrow) and lymphocytes (arrowhead). (C) Increased apocrine secretion (arrowhead) and goblet cell hypertrophy (arrow). (D) Increased apocrine secretion with vacuolization of the respiratory epithelium (arrow) and loss of cilia.

Many of the cytokines, most notably BMP3, exhibited significant downregulation at several time points. BMP3 is a structurally divergent member of the BMP family whose role in bone growth and development remains poorly understood. Some authors have suggested that BMP3 acts as an inhibitor of BMP cytokine family activity. This theory has been supported by studies in BMP3 knockout mice. BMP3-deficient mice demonstrate a significant increase in bone mass, and mice with BMP3 overexpression exhibit delayed ossification with spontaneous rib fractures.^{33,34}

One limitation of this study is the lack of obvious osteogenesis or osteitis noted on histologic analysis. This is consistent, however, with prior observations in the mouse model.^{26,27} Further investigation with the addition of later time points is ongoing. It is possible that careful inspection at later time points may reveal bony changes in the mouse model that would reflect chronic or longstanding disease. In a previously described mouse model of chronic otitis

media, osteogenesis was noted only after several months of chronic mucosal inflammation.³⁵

Conclusion

In this study we demonstrated changes in regulation of several tissue remodeling cytokines and enzymes over time in the mouse model of acute allergic rhinitis. Notable upregulation of MMP8 was noted at 6 hours, significant upregulation of FGF3 was seen at 1 week, and significant downregulation of BMP3 and BMP5 was observed at 1 week. These changes in expression were dynamic over time, suggesting a complex and evolving process. Although they are key regulators of tissue remodeling in many normal and pathologic processes, it remains unclear what role the MMP, BMP, and FGF families may play in CRS-associated tissue remodeling. Further study of these modulators of tissue remodeling is necessary in order to develop novel therapeutic strategies aimed at halting and/or reversing osteogenesis, scarring, and fibrosis in CRS. 4

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