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Steroid-independent upregulation of MMP9 in chronic rhinosinusitis patients with radiographic evidence of osteitis

Kara Y. Detwiler, MD¹, Timothy L. Smith, MD, MPH¹, Jess C. Mace, MPH¹, Dennis R. Trune, PhD², and Nathan B. Sautter, MD¹

¹Division of Rhinology and Sinus Surgery, Oregon Sinus Center, Department of Otolaryngology – Head and Neck Surgery; Oregon Health and Science University; Portland, Oregon.

²Oregon Hearing Research Center, Department of Otolaryngology – Head and Neck Surgery; Oregon Health and Science University, Portland, Oregon.

Abstract

Background—Chronic sinonasal inflammation is associated with tissue remodeling, such as osteitis, which may be a marker of refractory disease; however, the pathophysiology of osteitis in chronic rhinosinusitis (CRS) is insufficiently understood.

Methods—Ethmoid mucosa and bone samples were obtained from 35 medically refractory CRS patients and 9 control subjects. Quantitative real time polymerase chain reaction (RT-PCR) was performed separately on bone and mucosa for Matrix Metalloproteinase 2 and 9 (MMP2, MMP9) and tissue inhibitor of matrix metalloproteinase 1 (TIMP1). Osteitis was classified as mild, moderate, or severe by measuring bone thickness of the maxillary, sphenoid, and ethmoid sinuses on multiplanar CT. Patients were classified based on severity of osteitis and compared to controls.

Results—9 patients demonstrated radiographic evidence of osteitis (mild=3, moderate/severe=6). Bone PCR revealed biologically significant upregulation of MMP9 in all patients with CRS, but the magnitude of the upregulation decreased with severity of osteitis. Mucosa PCR showed upregulation of MMP9 in moderate/severe osteitis only. No significant changes were seen in MMP2 or TIMP1 regulation.

Conclusion—This is the first study to evaluate the role of MMP in the bone and mucosa of patients with sinonasal osteitis. The pattern of expression suggests there may be a time and tissue dependent role for MMP9 in the pathophysiology of osteitis. In addition, MMP9 overexpression is seen despite preoperative oral and intranasal steroid use, suggesting that if MMP9 is an important factor in the development of osteitis then steroids may not be the best treatment in prevention of osteitis.

Index Medicus

Osteitis; MMP9; matrix metalloproteinases; chronic rhinosinusitis; steroids; computed tomography

Corresponding Author: Nathan B. Sautter, MD, Department of Otolaryngology – Head and Neck Surgery, Oregon Health and Science University, 3181 SW Sam Jackson Park Road, PV-01, Portland, OR 97239, 503-418-9691 (Fax), sauttern@ohsu.edu.

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Introduction

Tissue remodeling is a dynamic molecular process of formation and resorption leading to transient or permanent changes in tissue architecture.¹ Tissue remodeling of the lower airway has been extensively studied in asthmatics and is associated with disease severity.^{2,3} More recently, the “unified airway” theory has led to increased interest in sinonasal tissue remodeling secondary to chronic rhinosinusitis (CRS)^{4,5}, but the underlying molecular mechanisms leading to these changes are unknown.

In CRS, tissue remodeling is exhibited in both mucosa and bone, characterized by osteitis, mucosal hypertrophy, fibrosis, and thickening of the basement membrane.¹ Tissue remodeling affects normal sinonasal physiology and may worsen disease severity.⁵ Although mucosal remodeling in CRS has been investigated, the role of bone remodeling, or osteitis, has only recently become of interest and may play an equally important role. Osteitis is described in general terms as inflammation of the bone, and several studies have demonstrated the presence of osteitis in CRS.^{6,7} Histologically, osteitis is a combination of periosteal thickening, fibrosis, osteosclerosis and osteolysis often resulting in replacement of well-organized lamellar bone with immature woven bone.⁷ Although histologic comparisons have been drawn between osteomyelitis of the long bones and osteitis of the sinonasal bones, the pathophysiology appears to be different. While osteomyelitis is a purely infectious process, osteitis appears to be a predominantly inflammatory process. Although infection may contribute to the development of osteitis, there are no studies demonstrating a significant role for bacteria in osteitis.⁷ Furthermore, microcolonies of bacteria have been observed within the sphenoid bones of both CRS patients and healthy controls with no correlation between bone thickness and presence of bacteria.⁸ It is postulated that bacterial inoculation and/or uncontrolled inflammation from the mucosa spreads to the bone and propagates widely via Haversian canals. This hypothesis is supported by animal studies in which bone changes were seen in the non-inoculated sinuses.⁹ Regardless of the inciting event, osteitis is theorized to disseminate inflammation to the overlying mucosa, potentially resulting in chronic inflammation and recalcitrant disease. This theory is supported by the anecdotal finding that debridement of osteitic bone can resolve persistent inflammatory disease in CRS patients undergoing revision endoscopic sinus surgery (ESS), while debridement of only the mucosa often has no effect on reducing overall inflammation.⁶ In addition, radiographic evidence of osteitis has been associated with worse baseline disease severity and treatment outcomes.^{10,11} As osteitis may be associated with recalcitrant disease, improved understanding of the underlying molecular mechanisms leading to these tissue changes warrants further investigation and could lead to novel therapies. This is of particular importance when considering the theory that, over time, tissue remodeling in CRS reaches a point at which it becomes irreversible, leading the patient down the path of refractory disease.⁵

Although bone remodeling is governed by many factors, matrix metalloproteinases (MMP) are of particular interest given their role in tissue remodeling. MMPs are among the most well studied tissue remodeling factors in CRS, yet have never been investigated in association with osteitis. In humans, the MMP family is comprised of 24 proteolytic enzymes that regulate remodeling of the extracellular matrix throughout the body. In addition to their remodeling functions, MMPs are involved in inflammatory pathways, cell recruitment, and immune response.¹² MMP dysregulation has been demonstrated in a murine model of CRS.¹³ In humans, MMP dysregulation has also been linked to CRS as well as other inflammatory diseases such as otitis media and asthma.¹³ Of particular interest in CRS are MMP2, MMP9, and tissue inhibitor of matrix metalloproteinases-1 (TIMP1)^{12,14–17}; however, the relationship between these tissue remodeling factors and osteitis has never been explored. We hypothesize that these tissue remodeling factors are

involved in the development of osteitis, and the purpose of our study was to evaluate the expression of MMP2, MMP9, and TIMP1 in the bone and mucosa of patients with CRS.

Materials and Methods

Study Population and Inclusion Criteria

The study population consisted of 35 adult subjects with CRS and nine control patients without CRS, allergy, or recurrent acute sinusitis. All CRS patients met diagnostic criteria for chronic rhinosinusitis as described by Multi-Disciplinary Sinusitis Guidelines.^{18,19} All subjects elected to pursue ESS after symptoms persisted following standard initial medical management consisting of a three-week course of culture-directed or broad-spectrum antibiotics, a tapering course of oral prednisone, nasal saline irrigations, and an eight-week course of topical nasal steroid spray. Both primary and revision cases were included in this study. All study patients were placed on perioperative steroids and antibiotics a week before surgery. Broad spectrum antibiotics were continued for a total of two weeks. Prednisone was initiated at 30 mg daily and tapered over 12 days. The control subjects for this study consisted of nine patients without a diagnosis of CRS, recurrent acute rhinosinusitis or inhalant allergy who were undergoing endoscopic surgery for treatment of nasal obstruction secondary to concha bullosa or transsphenoidal approach to the pituitary gland. Voluntary informed consent was obtained from all participants and the Institutional Review Board (IRB) at OHSU provided approval and oversight for this research.

Diagnostic Criteria for Radiographic Osteitis

Standard preoperative computed tomography (CT) of the sinuses were obtained while the patients were undergoing standardized medical treatment for CRS. Using axial cuts with multiplanar reconstructions, the CT scans were reviewed in a blinded, retrospective fashion using image management software (Impax, AGFA Healthcare, Greenville, SC) by a single Otolaryngologist. The bone thickness of the maxillary, sphenoid, and ethmoid sinuses were systematically measured in axial and coronal planes at regular intervals in a standardized fashion (Figure 1). Osteitis was diagnosed when the bone thickness measured at least 3 mm, and the degree of osteitis was classified as mild (>3–4 mm), moderate (>4–5mm), or severe (>5mm) as previously described.²⁰ Patients with CRS were categorized according to severity of radiographic osteitis and compared to controls.

Specimen Collection

Bone and mucosal biopsies were taken intraoperatively from the ethmoid bulla of study patients and concha bullosa or sphenoid bone of control patients. The mucosa was separated from the bone and the tissues were preserved immediately in RNAlater (Applied Biosystems, Carlsbad, CA). The tissue samples were stored at –80°C until they were processed for RNA extraction.

Reverse Transcription Polymerase Chain Reaction (RT-PCR)

RNA for RT-PCR was extracted from the mucosa and bone individually using the RNeasy Mini Kit according to the manufacturer's instructions (Qiagen, Inc.). A total of 5 µg mRNA was used to synthesize a cDNA probe using the RT² first strand kit (SABiosciences, Frederick, MD) for hybridization to a custom made human gene array membrane including MMP9 and GAPDH as a housekeeping control (Applied Biosystems, Carlsbad, CA). The method of semiquantitative real-time RT-PCR was utilized performed using an ABI Step One Plus system (Applied Biosystems, Inc., Carlsbad, 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.

Calculation of the fold change was performed using the $\Delta\Delta C_t$ method with the aid of SABiosciences PCR Array Data Analysis Web Portal. According to the manufacturer, biologically significant fold change is defined as greater than or equal to 2.0 or less than or equal to 0.5.

Results

Patient Characteristics

Nine patients with CRS (26%) demonstrated radiographic evidence of osteitis. Three patients had mild osteitis and six had moderate or severe osteitis. Of the osteitis patients, three (33%) had CRS with nasal polyposis and three (33%) were revision cases. Of the patients without osteitis, nine (32%) were revision cases and eight (29%) had CRS with nasal polyposis. We did not observe any differences in other baseline disease characteristics: three patients with osteitis (33%) had asthma, and three (33%) had documented allergy. In regards to patients without osteitis, twelve (43%) had asthma, and eight (28%) had documented allergy. The average age of the osteitis patients was 49.4 years, and the average age of the patients without osteitis was 47.5 years. No patients were prescribed long-term macrolide therapy.

RT-PCR

Bone

MMP9: Patients without osteitis demonstrated a biologically significant 3.13 fold upregulation of MMP9 as compared to controls. All patients with osteitis demonstrated a biologically significant 2.64 fold upregulation of MMP9, while patients with moderate/severe osteitis had a biologically significant 2.35 fold upregulation. (Table 1)

MMP2: There were no biologically significant changes in regulation between control patients and patients without osteitis (fold change=0.88), all patients with osteitis (fold change=0.69), or patients with moderate/severe osteitis (fold change=0.52).

TIMP1: There were no biologically significant changes in regulation between control patients and patients without osteitis (fold change=1.38), all patients with osteitis (fold change=1.27), or patients with moderate/severe osteitis (fold change=1.15).

Mucosa

MMP9: There were no biologically significant changes in regulation between control patients and patients without osteitis (fold change=1.76) or all patients with osteitis (fold change=1.72). Patients with moderate/severe osteitis had a biologically significant 3.20 fold upregulation (Table 1).

MMP2: There were no biologically significant changes in regulation between control patients and patients without osteitis (fold change=0.65), patients with osteitis (fold change=0.78), or patients with moderate/severe osteitis (fold change=0.75).

TIMP1: There were no biologically significant changes in regulation between control patients and patients without osteitis (fold change=0.96), patients with osteitis (fold change=0.82), or patients with moderate/severe osteitis (fold change=0.79).

Discussion

We believe this is the first study to investigate the association between MMP2, MMP9, TIMP1, and osteitis. In addition, this is the first study to evaluate differential gene expression in the bone and mucosa of patients with CRS.

We observed biologically significant upregulation of MMP9 in the bone of all CRS patients. However, the magnitude of upregulation decreased as the severity of radiographic osteitis increased. The mucosa showed biologically significant upregulation of MMP9 in moderate/severe osteitis only. There were no biologically significant fold changes in MMP2 or TIMP1 expression. Although alterations in MMP2 and TIMP1 have been seen in CRS with nasal polyposis (CRSwNP) and CRS without nasal polyposis (CRSSNP), these remodeling factors do not appear to play a significant role in osteitis.^{12,17}

MMP9 is a collagenase, and one of its main functions is to breakdown type IV collagen which is the major structural component of the basement membrane. MMP9 also assists in the transepithelial migration of inflammatory cells. Elevated levels of MMP9 have been found in both CRSwNP and CRSSNP¹², and MMP9 polymorphisms may be a risk factor in the development of CRS.²¹ In addition, increased MMP9 has been linked with poor surgical healing.²² Our findings support a role for MMP9 in CRS and may suggest a time and tissue dependent role for MMP9 in the development of osteitis.

Bone remodeling may be conceptualized as a time-dependent process where patients with more severe osteitis are likely those with long-standing uncontrolled inflammation. If this is true, our findings suggest that MMP9 overexpression in bone may be important early in the development of osteitis whereas overexpression in the mucosa is observed later in the course of osteitis. (Figure 2) In addition, MMP9 overexpression was observed despite oral and intranasal steroid therapy, suggesting that MMP9 gene regulation may not be completely steroid responsive. This is consistent with a previous study in which oral prednisolone did not reduce the levels of MMP9 in nasal secretions. Interestingly, this study also showed that doxycycline reduced MMP9 levels.²³ In addition to doxycycline, macrolide antibiotics have been shown to inhibit MMP9 in vivo and perhaps there is role for these therapies in preventing osteitis.²⁴ We were unable to investigate this further as none of the patients in our current study were treated with doxycycline or macrolide antibiotics.

There are limitations to this study. The patient population is small given the heterogeneity of CRS. This must also be considered in light of the wide variation in gene expression observed. Radiographic evidence of osteitis is not as sensitive as histological evidence of osteitis²⁰ and therefore may not capture all patients with osteitis. Other studies have measured osteitis in Hounsfield Units²⁵ or using single photon emission computed tomography bone scintigraphy^{10,26} and these methods could be considered in future investigations. Additionally, advances in volumetric imaging techniques may allow for increasingly accurate and precise quantification of osteitis. We are currently in the process of evaluating the surgical pathology from all study participants and plan to reanalyze the PCR results once this data is available. All study patients received preoperative antibiotics and steroids in an effort to control mucosal inflammatory disease and prepare the sinonasal mucosa for surgical intervention, while control patients did not receive this regimen. This potential limitation is difficult to overcome in our current observational study design; however, we plan to further investigate this in the future by specifically identifying and evaluating patients who cannot tolerate systemic steroids (e.g. diabetic patients). Alternatively, a preoperative tissue biopsy (prior to initiation of steroid therapy) could be performed in clinic, although patient discomfort may limit this practice. Another alternative may be in-office tissue brushing with cell culture prior to initiation of antibiotics. We found

it particularly interesting that Van Zele et al. observed no impact of oral steroids on MMP9 while doxycycline and macrolides reduced levels of MMP9.²⁴ This highlights the need to better understand the impact of various drug classes, beyond steroids, on MMP9 and other gene expression in CRS.

In summary, osteitis is seen in CRS and may be associated with recalcitrant disease; however, the molecular mechanisms leading to osteitis are unknown. Further knowledge of the pathophysiology of osteitis could lead to improved or novel medical therapies and augment our understanding of recalcitrant sinusitis.

Conclusion

This is the first study to evaluate the role of MMP in CRS patients with sinonasal osteitis. The pattern of expression suggests there may be a time and tissue dependent role for MMP9 in the pathophysiology of osteitis such that bone MMP9 expression is increased in the early phases of disease while mucosal MMP9 expression is increased in later phases of disease. In addition, MMP9 overexpression is observed despite preoperative oral and intranasal steroid use, suggesting that if MMP9 is an important factor in the development of osteitis then steroids may not be the best treatment in prevention of osteitis.

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Figure 1.
Example of severe radiographic osteitis of the sphenoid sinus. Black arrow signifies measuring caliper used to determine bone thickness.

Figure 2.

The role of MMP9 in sinonasal osteitis may be tissue and time dependent. In this schematic, severity of osteitis is represented on the x-axis and level of MMP9 expression is shown on the y-axis. Level of MMP9 expression in bone as it relates to osteitis severity is represented by the blue line, and MMP9 expression in mucosa is denoted by the red line. The black line represents normal baseline MMP9 expression. **It is posited that osteitis severity increases with time, or length of disease, and therefore the x-axis may also potentially be labeled as a function of time.**

Table 1

Change in regulation of MMP9 in bone and mucosa of CRS patients with and without osteitis compared to control patients.

	Fold Change		
	No Osteitis	All Osteitis	Moderate/Severe Osteitis
Bone	3.13 *	2.64 *	2.35 *
Mucosa	1.76	1.72	3.20 *

Biologically significant upregulation, as defined by a fold change >2.0, is indicated by an asterisk (*).