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Epigenetics of Chronic Rhinosinusitis and the Role of the Eosinophil

Kristin A Seiberling, MD, Christopher A Church, MD, Jason Herring, PhD, and Lawrence Sowers, PhD

Loma Linda University Department of Otolaryngology-Head and Neck Surgery

Abstract

Introduction—One theory for the pathogenesis of chronic rhinosinusitis with nasal polyps (CRSwNP) involves aberration in the expression of genes that maintain the sinonasal innate immune system. We propose that the alteration in gene expression seen in CRSwNP is a result of oxidative byproducts of eosinophils. Activated eosinophils and neutrophils may lead to the production of hypobromous acid (HOBr) and hypochlorous acid (HOCL) and the post-translational modification product 5-bromocytosine (5BrC) and 5 chlorocytosine (CIC) respectively. 5BrC and CIC may cause aberrant methylation of cytosine during DNA replication and mimic the endogenous methylation signal associated with gene silencing. We propose to use gas chromatography-mass spectrometry (GC-MS) to identify the presence of 5BrC and CIC in CRSwNP patients.

Methods—Patients with CRSwNP undergoing endoscopic sinus surgery were prospectively recruited into this study. Using GC-MS tissue specimens were analyzed for the presence of 5BrC, CIC and methylated cytosine.

Results—Tissue specimens from 14 patients with CRSwNP and 3 normal controls were processed using GC-MS. CRSwNP specimens demonstrate elevated levels of 5BrC and CIC compared to normal controls.

Conclusion—Eosinophils, which are predominantly found in CRSwNP, may lead to DNA modification and gene silencing via 5BrC and aberrant methylation patterns and may help explain the pathogenesis of CRSwNP.

INTRODUCTION

Chronic rhinosinusitis (CRS) is one of the most prevalent diseases in the United States with over 15% of the general population affected ¹. The term CRS covers a spectrum of diseases; however, two major forms can be differentiated, CRS with nasal polyps (CRSwNP) and CRS without nasal polyps (CRSsNP). CRSwNP is characterized by polypoid edema, tissue eosinophilia and is dominated by Th2 cytokines ². Up to 70–90% of patients with CRSwNP demonstrate abundant tissue eosinophilia ³, which is absent to a large degree in CRSsNP.⁴. Eosinophils release toxic mediators such as eosinophilic cationic protein (ECP), eosinophilic

peroxidase (EPO) and major basic protein (MBP), which are known to damage the respiratory mucosa⁵⁻⁹. EPO and myeloperoxidase (from neutrophils) are capable of generating hypobromous acid (HOBr)¹⁰ and hypochlorous acid (HOCl) respectively¹¹. These hypohalous acids function well as antimicrobial agents; however, they can also cause substantial collateral damage to normal host tissues. The hypohalous acids are capable of oxidatively modifying target protein and lipids within inflamed tissue^{12,13}. Alternatively, the hypohalous acids may react with DNA, resulting in an array of damaged bases including 5-chlorocytosine (CIC) and 5-bromocytosine (5BrC)¹⁴⁻¹⁶ depicted in Fig. 1. These modified forms of cytosine (5BrC and CIC) may result in the aberrant methylation of cytosine during DNA replication. Cytosine methylation is a post-replicative enzymatic process that occurs predominantly in the CpG dinucleotide islands located within the promoter region of genes¹⁷. Cytosine methylation patterns in DNA comprise an epigenetic code for controlling gene activity and have been linked to differential gene expression^{18,19}. Methylation of cytosine can lead to transcriptional silencing of genes and altered gene expression without changes in the genomic sequence, whereas the absence of cytosine may lead to gene transcription.

In vitro studies have demonstrated that methylation-sensitive DNA-binding proteins cannot distinguish 5-halocytosines from 5-methylcytosine (5mC), binding both with high affinity^{20,21}. These 5-halocytosines may be as good as or even better than 5mC in directing DNA methylation of the target cytosine in the CpG sequence context. Halogenated cytosine residues also bind human maintenance methylase with a high affinity, allowing the aberrant methylation pattern to be propagated through DNA replication²². Furthermore, these 5-halocytosines appear not to be recognized as damaged bases by DNA repair glycosylases, allowing for the accumulation of these analogues within the genome^{20,23}. Therefore, 5BrC can mimic the endogenous methylation signal associated with gene silencing, resulting in altered gene expression which may be preserved during DNA replication.

Inflammatory cell infiltrates are characteristically found in both CRSsNP and CRSwNP suggesting that inflammation may influence disease pathogenesis. However, the role of both neutrophils and eosinophils in the etiology of CRSwNP is yet to be established. We hypothesize that eosinophils, which are predominantly found in CRSwNP, may lead to DNA modification and gene silencing through the production of 5BrC and may explain the difference in presentation between CRSwNP and CRSsNP. In this study, our goal is to determine the presence of 5BrC, CIC and methylated cytosine in CRSwNP compared to controls.

METHODS

Patient selection

After approval from the Loma Linda University Institutional Review Board, patients between the ages 16 and 80 years were prospectively recruited for this study. Patients were selected into two groups, CRSwNP and normal controls. CRS was diagnosed as outlined by the Sinus and Allergy Health Partnership²⁴. Only those who failed medical management and required endoscopic sinus surgery were included in the study. Nasal polyps were confirmed by nasal endoscopy and computed tomography (CT). Patients were excluded if

they had a history of allergic fungal sinusitis, atopy or allergic rhinitis, Samter's triad, cystic fibrosis, known immune deficiency, Kartagener's syndrome, inverted papilloma, sinonasal tumor or antrochoanal polyp. Patients selected for the control group were undergoing endoscopic transphenoidal surgery for benign pituitary tumors and did not have symptomatology, imaging or endoscopic findings consistent with CRS. Controls with allergic rhinitis were also excluded.

Specimen collection

Sinonasal tissue was obtained at the time of endoscopic sinus surgery. One gram of tissue was collected and stored. In the CRSwNP cohort, nasal polyps were obtained and processed. Control tissue was obtained from the posterior ethmoid cells. All tissue specimens were stored at -80 degree Celsius until further processed.

DNA extraction

Samples were thawed on ice and approximately 25–50 mg of each sample was removed and cut into smaller pieces and placed into a 1.5 ml Eppendorf tube. DNA was extracted using the Qiagen DNAeasy kit for tissue.

DNA hydrolysis and preparation for mass spectrometry

2µg of DNA was added to vials with conical inserts and dried under reduced pressure. Next, 100µl of 88% formic acid was added to samples, and the vials were capped and heated for 40 minutes at 140 degree Celsius. Samples were cooled and dried under reduced pressure prior to derivatization. Samples were derivatized for mass spectrometry by making their tert-butyl dimethylsilyl derivatives. This was done by adding 20µl dry acetonitrile and 20µl MTBSTFA to the dry sample, then capping and heating at 140 degree Celsius for 40 minutes.

Mass spectrometry analysis

Samples were injected into the mass spectrometer to identify tert-butyl derivatives of 5BrC. The tert-butyl derivatives for 5BrC and CIC have masses of 360–362 m/z and 316–318m/z, respectively. Retention times for 5BrC and CIC are 12.82 and 12.48 minutes, respectively. During mass spectrometry, analysis samples were monitored in SIM mode looking for the characteristic peaks (Figure 2).

Statistical analysis

Statistical analysis was carried out using PRISM GraphPad software (La Jolla, California). Data sets were analyzed using an unpaired T test. Statistical significance was determined by a p value < 0.05.

RESULTS

Seventeen patients, 14 with CRSwNP and 3 normal controls were included in this study. Those with CRSwNP ranged in age between 16–79 (average 47.5) years. The ages of the control patients are 16, 52 and 55 years. There were 8 males and 6 females in the CRSwNP group and 2 males and 1 female in the control group. The average Lund Mackay score in the

CRSwNP group was 14.5 (range 8–24). Ten patients in the CRSwNP underwent revision sinus surgery with an average of 1.66 previous surgeries (range 1–5). Seven out of the 14 patients with CRSwNP had asthma and none of the controls. All patients were non smokers. Data obtained from CRSwNP and normal controls is presented in Table 1 and plotted in Fig. 3. The level of 5BrC in the polyp tissue (CRSwNP) was significantly higher than that in the normal controls ($p=0.007$), and the average level was approximately 5 times that observed in the normal controls. Levels of 5CIC in polyp tissues were also slightly elevated when compared to controls although this level did not reach significance ($p=0.66$). The levels of 5CIC and 5BrC are compared in Fig. 4. 5-halocytosine levels in CRSwNP patients on preoperative oral or nasal corticosteroids were not significantly different from those not on these medications. No significant differences between CRSwNP and controls were noted in 5mC levels. In the CRSwNP group, 7 patients were placed on preoperative oral prednisone (40 mg taper starting one week prior to surgery), 2 on preoperative nasal steroid sprays and 1 on both oral and nasal steroid sprays. There was no statistical difference between the levels of 5BrC and CIC in those patients with polyps on and off oral steroids (Figure 5). There was also no difference in the levels of CIC or 5BrC between those with and without asthma.

DISCUSSION

In a previous study, it was demonstrated that both 3-chlorotyrosine and 3-bromotyrosine were elevated in proteins extracted from diseased mucosal tissues, confirming the participation of the hypohalous acids in tissue damage associated with CRSsNP and CRSwNP²⁵. In this study, we report the presence of similar byproducts of hypohalous acids, CIC and 5BrC, in nasal polyp tissue. These halogenated pyrimidine lesions result from peroxidase-mediated inflammatory processes and are known to influence epigenetic control by altering DNA methylation patterns^{26, 27}. Much of the current literature deals with inflammation-mediated cytosine damage on human cancer cell lines. In human tumors, it is hypothesized that tissue inflammation leads to local hypermethylation in the promoter region of tumor suppressor genes leading to gene inactivation and growth of cancer cell lines. Looking at DNA methylation patterns in human cancer lines, Paz et al demonstrated tumor-specific patterns of hypermethylation in the CpG islands located within the promoter region (local hypermethylation) in the context of generalized global genomic hypomethylation^{28,29}. *In vitro* work has demonstrated that the inflammation-mediated DNA damage products CIC and 5BrC can direct fraudulent methylation of previously unmethylated CpG sites and alter gene expression by epigenetic changes³⁰. Furthermore, in this study it has been shown that CIC is incorporated into DNA and results in increased levels of methylation within the promoter region of a targeted sequenced gene.

To our knowledge, this study is the first report of the measurement of 5CIC and 5BrC in human sinonasal tissue *in vivo*. The results obtained are consistent with collateral inflammatory damage to sinonasal mucosal tissue. These results favor the hypothesis that CRSwNP pathogenesis is a result of inflammatory changes and dependent on eosinophilic, and to a lesser degree, neutrophilic inflammation. Furthermore, these results give us insight into the possible role of the eosinophil in modifying target DNA leading to epigenetic changes and disease formation. This is a pilot study with encouraging results however

further studies with larger numbers will be required to understand the biological significance of the halocytosines in DNA tissues.

CONCLUSION

Epigenetic aberrations that influence innate epithelial immunity as a result of activated eosinophils may play a major role in the pathogenesis of CRSwNP and account for the difference in disease presentation between CRSwNP and CRSsNP. Further studies are needed that define the role of 5BrC and CIC in the pathogenesis of CRS.

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Figure 1.
Structures of Cytosine (C), 5-Chlorocytosine (5ClC) and 5-Bromocytosine (5BrC)

Figure 2. GC/MS of polyp 5ClC and 5BrC

The extracted ion chromatogram for the m and m+2 ions for the TBDMS derivatives (316/318 and 360/362 m/z respectively) of 5ClC and 5BrC in polyp DNA (A), have the same isotopic abundance ratio as the derivatized free base standards (B). The isotopic abundance ratio between the m and m+2 ions for 5ClC and 5BrC are unique and diagnostic of the presence of chlorine and bromine.

Figure 3. 5-Bromocytosine and 5-Chlorocytosine DNA content in Human Nasal Polyp and Normal Tissue

Following acid hydrolysis and GC-EI-MS analysis the DNA 5BrC content in samples (—) and their averages (○) were plotted to compare 5BrC (A) and 5ClC (B) levels in CRS with nasal polyp (CRSwNP) and normal non-CRS non-polyp control surgical specimens. The average 5BrC content in CRSwNP DNA (260 5BrCs/10⁶ Cs) was more than 4 times higher than non-CRS non-polyp controls (65 5BrCs/10⁶ Cs). The average 5ClC content in CRSwNP and non-CRS non-polyp control DNA was 308 5ClCs/10⁶ Cs and 248 5ClCs/10⁶ Cs respectively.

Figure 4. Comparison of 5CIC and 5BrC DNA Content

To examine the relationship between DNA 5BrC and 5CIC content, 5CIC content was plotted against 5BrC content. Neutrophil mediated inflammation generates both 5CIC and 5BrC while eosinophil mediated inflammation generates predominately 5BrC; therefore, samples with higher amounts of 5CIC suggests a greater neutrophil involvement in inflammation damage.

Figure 5.

Table 1

Halocytosine Content in Polyp and Normal Human Samples

Sample	5mC/C%	5BrC/10 ⁶ Cs	5ClC/10 ⁶ Cs	Group
1	4.2% ± 0.05%	531 ± 118	710 ± 30	CRSwNP
2	4.0% ± 0.02%	273 ± 89	166 ± 29	CRSwNP
3	3.8% ± 0.01%	269 ± 52	720 ± 51	CRSwNP
4	4.2% ± 0.05%	136 ± 52	57 ± 50	CRSwNP
5	4.1% ± 0.20%	148 ± 1	242 ± 11	CRSwNP
6	4.0% ± 0.02%	152 ± 10	131 ± 27	CRSwNP
7	3.9% ± 0.05%	294 ± 108	302 ± 153	CRSwNP
8	4.2% ± 0.04%	307 ± 22	313 ± 84	CRSwNP
9	4.2% ± 0.02%	198 ± 35	295 ± 61	CRSwNP
10	3.8% ± 0.04%	326 ± 42	279 ± 90	CRSwNP
11	4.5% ± 0.13%	324 ± 51	319 ± 129	CRSwNP
12	4.3% ± 0.02%	287 ± 15	423 ± 34	CRSwNP
13	4.1% ± 0.05%	151 ± 13	97 ± 30	CRSwNP
14	4.5% ± 0.47%	236 ± 12	264 ± 43	CRSwNP
15	3.9% ± 0.02%	50 ± 10	160 ± 25	control
16	4.3% ± 0.10%	61 ± 9	264 ± 33	control
17	4.1% ± 0.13%	86 ± 20	321 ± 93	control