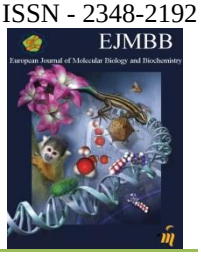
e - ISSN - 2348-2206
Print ISSN - 2348-2192

European Journal of Molecular Biology and Biochemistry

Journal homepage: www.mcmed.us/journal/ejmbb



INVESTIGATING HMGB1 PROTEIN IN CHRONIC RHINOSINUSITIS WITH NASAL POLYPS: AN IN-DEPTH IMMUNOHISTOLOGICAL ANALYSIS

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Article Info

Received 20/12/2015
Revised 16/01/2016
Accepted 24/01/2016

Key words:-
Chronic rhinosinusitis with nasal polyps (CRSwNP), HMGB1 protein, Inflammation pathogenesis, ENT pathology, Immunohistochemistry.

ABSTRACT

Background: Chronic rhinosinusitis with nasal polyps (CRSwNP) is a complex inflammatory condition influenced by various immune and environmental factors. The nuclear protein HMGB1 has been implicated in inflammation pathogenesis, with its role in CRSwNP remaining under investigation. Objective: This collaborative study aimed to explore HMGB1 expression in CRSwNP patients and its association with inflammatory processes, particularly eosinophilic involvement, within the ENT region. Methods: Between 2014 and 2016, 42 nasal polyp tissue specimens were collected at Prathima Institute of Medical Sciences, Nagunoor, Telangana, India. Participants included healthy controls, asthmatics, and patients with allergic rhinitis. Diagnoses followed the European Position Paper on Rhinosinusitis and Nasal Polyps criteria. Clinical assessments, including symptom severity, endoscopic evaluations, and CT scans, were conducted. Immunohistochemical analysis of biopsy specimens assessed HMGB1, IL-5, and IL-16 expression. Statistical analyses were performed to correlate HMGB1 expression with eosinophilic infiltration and other inflammatory markers. Results: CRSwNP patients exhibited higher HMGB1 expression compared to controls, with significant localisation differences in epithelial cytoplasm, epithelial nuclei, and subepithelial areas. Eosinophilic CRSwNP showed increased HMGB1 in inflammatory cells but reduced subepithelial infiltration compared to non-eosinophilic cases. HMGB1 expression was associated with IL-5, IL-8, and TNF-α, indicating its role in CRSwNP pathogenesis. Conclusion: HMGB1 plays a crucial role in the inflammatory mechanisms of CRSwNP, particularly in eosinophilic infiltration. These findings enhance our understanding of CRSwNP pathogenesis and highlight potential therapeutic targets. Continued research on HMGB1 in ENT pathologies could improve diagnostic and treatment strategies for chronic rhinosinusitis with nasal polyps.

INTRODUCTION

Various conditions can contribute to the development of chronic rhinosinusitis, including the formation of nasal polyps. Rhinosinusitis, whether viral, bacterial, or fungal, may arise as a complication of cystic fibrosis or primary ciliary dyskinesia [1]. Allergies, medication intolerances, gastroesophageal reflux disease (GERD), environmental pollutants, and adverse drug reactions can lead to an overactive immune response. Such cases are characterised by subepithelial oedema, epithelial damage, infiltration of eosinophils, mast cells, macrophages, and neutrophils (primarily eosinophils), along with the accumulation of inflammatory cells [2].

The release of mediators and cytokines by inflammatory cells is a normal physiological response to infections and tissue injury, highlighting inflammation as a natural defence mechanism. However, the amplification of the inflammatory response is driven by the recruitment

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and retention of immune cells. Several inflammatory diseases have been associated with the protein HMGB1 [3]. Specific membrane receptors play a role in activating necrotic or damaged tissues, as well as immune-activated tissues, leading to the release of inflammatory mediators. These mechanisms protect eosinophils, primarily by promoting endothelial activation and extending their survival [4].

This evolutionarily conserved protein functions as an "alarmin" in leukocytes and epithelial cells, residing in granules, the cytoplasm, and the nucleus. HMGB1, recognised as a late mediator of inflammation, is actively secreted by macrophages and monocytes into the extracellular environment. Additionally, exogenous pro-inflammatory agents, such as endotoxins, can stimulate macrophages and monocytes to release HMGB1 actively. Damaged or necrotic cells, including epithelial cells, can also release HMGB1 passively. When HMGB1 enters the surrounding environment, it diffusely activates endothelial cells [5].

Recent studies have demonstrated that nasal polyp (NP) tissue exhibits elevated levels of NF-kB compared to non-polyp nasal mucosa. NF-kB facilitates the transcription of cytokines, chemokines, and adhesion molecules through its signalling pathway, which carries significant implications for both disease progression and treatment. In addition to its involvement in NP management, transcription factors play a key role in the action of corticosteroids. HMGB1 may contribute to both allergic and non-allergic nasal mucosal inflammation. Persistent inflammation of the sinuses and nasal cavity, driven by chronic exposure to HMGB1, TNF, and IL, may lead to a sustained inflammatory state.

MATERIALS AND METHODS

This collaborative study, conducted across multiple countries and universities, investigated a nuclear protein associated with the pathogenesis of inflammation. The initial phase centred on a prevalent ENT pathology, with ongoing research examining inflammatory mechanisms within the ENT region. Between 2014 and 2016, 42 nasal polyp tissue specimens from patients with chronic rhinosinusitis with nasal polyps (CRSwNP) were collected at Prathima Institute of Medical Sciences, Nagunoor, Telangana, India. The study population included healthy controls, patients with asthma, and those with allergic rhinitis. Diagnoses were confirmed through a combination of medical history, nasal endoscopy, and computed tomography (CT) scans, adhering to the criteria outlined in the European Position Paper on Rhinosinusitis and Nasal Polyps 3. Atopic status was determined through skin prick testing for aeroallergens. Symptom severity was rated on a 0–10 scale, and the Lund-Mackay classification was applied to CT findings. Exclusion criteria encompassed specific medical conditions that might confound the results. Pre-surgical biopsy samples were evaluated using the Lund Kennedy system and underwent immunohistochemical analysis for HMGB1, IL-5, and IL-16. Frozen tissue samples were subsequently analysed to provide detailed insights into the clinical features of both patients and controls [6].

Using hematoxylin staining, the study identified 16 controls, 20 CRSwNP patients with eosinophilia, and 22 CRSwNP patients without eosinophilia. The degree of eosinophilic infiltration was assessed in 20 high-power (HP) fields under x400 magnification. Patients with an eosinophil count exceeding 20 per HP field were classified as Eos CRSwNP. Results comparing eosinophilic and non-eosinophilic CRSwNP groups are presented in Table II.

Immunohistochemical staining

A cryostat was used to section biopsy samples (7m) after fixation and dehydration. After air drying for 10 minutes, sections were permeabilized in PBS and placed on chromegelatin-coated slides. Endogenous peroxidases were blocked by applying 3% H2O2 to the skin for 10 minutes at room temperature in the dark. To prepare the slides for blocking, the slides were rinsed in PBS three times for 2 minutes each before being blocked for 20 minutes in PBS containing 10% normal horse serum. Incubation was carried out overnight at 4° with rabbit polyclonal antibodies (ab-18256, dilution 1:300), mouse monoclonal antibodies (ab25034, dilution 1:200), mouse monoclonal antibodies (ab1793, dilution 1:200) and goat polyclonal antibodies (ab10769, dilution 1:200). A five-minute rinse in PBS was followed by three rinses in PBS [7]. As an alternative, secondary antibodies were prepared using PV-9003 or PV-9000 kits. ZSJQ kits provided two minutes of diaminobenzidine development. Mayer's haematoxylin was used to counterstain the slides and neutral resin was used to mount them. Using normal rabbit serum as the positive control, PBS was used as the negative control.

SPSS13.0 software was used to perform all statistical analyses. Except where otherwise noted, all using a repeated measures ANOVA with LDS posthoc multiple comparisons. In cases where there were differences between two groups, Mann-Whitney U tests were applied. Spearman rank correlation and Pearson correlation are both used to evaluate correlations. An analysis of multivariate regression was conducted to determine whether the expression of HMGB1 protein correlates with eosinophils, IL-5, IL-8, and TNF-□ positive cells. The significance of a p value in statistics was defined as less than 0.05.

Table 1: Describe the clinical characteristics of the study participants

	Control subjects			CRSw			NP	p value	
No. of subjects	16			20			22	–	
Sex (male/female)	5/3			8/2			8/3	NS	
Mean age(y), median range	37(22-70)			42 (30-54)			38 (17-56)	NS	
Nasal congestion	2			18			22	< 0.001	
Rhinorrhoea	0			18			18	0.001	
Headache	2			12			6	NS	
Facial pain/pressure	0			2			2	NS	
Hyposmia	0			16			12	0.014	
No. of bilateral polyps	0			20			8	NS	
Recurrent history	0			4			6	NS	
Atopy	Y	N	U	Y	N	U	Y N	U	NS
	0	8	8	4	10	6	0 10	12	
Asthma	0	16	0	2	18	0	0 22	0	
Increased eosinophils*		0			16		6		0.005
Increased lymphocytes*		0			4		2		NS

Table 2: By comparing CT scans and nasal endoscopic examinations, differences in symptoms between individuals with Eos CRSwNP and those without Eos CRSwNP were determined.

MS	3.30 ± 0.60	3.00 ± 0.68	NS
AES	4.10 ± 1.03	1.00 ± 1.61	NS
PES	3.50 ± 0.87	2.36 ± 1.29	0.041
SS	3.30 ± 2.03	0.61 ± 1.04	0.020
FS	3.50 ± 2.17	2.54 ± 1.43	NS0
OMCS	4.50 ± 0.60	3.55 ± 3.24	NS
ES	6.70 ± 2.75	4.37 ± 1.75	0.041
TS	26.50 ± 5.17	20.45 ± 6.84	0.010
Endoscopy	4.50 ± 2.26	4.55 ± 1.56	NS
Nasal congestion	8.5 ± 1.67	4.56 ± 1.58	NS
Rhinorrhoea	6.70 ± 1.53	6.81 ± 2.14	NS
Headache	4.50 ± 1.80	2.36 ± 2.21	0.041
Hyposmia	6.00 ± 3.16	4.35 ± 5.36	0.010

RESULTS

A nasal polyp from chronic rhinosinusitis eosinophilic patients, a chronic rhinosinusitis unaffected patient, and a nasal polyp from chronic rhinosinusitis naive patients, The immunohistochemistry staining of sections from patients with eosinophil chronic rhinosinusitis and nasal polyps, and patients without, CRSwNP patients and controls both showed immunohistochemical recognition of HMGB1. The staining was divided into four sections based on HMGB1 protein expression level: epithelial cytoplasm, epithelial nucleus, focal extracellular infiltration, and inflammation. CRSwNP patients with non-eosinophilic CRS expressed HMGB1 at higher levels than controls. The epithelial cytoplasm staining of HMGB1 was significantly reduced in patients with CRSwNP who were eosinophilic and non-eosinophilic. Subepithelial HMGB1 protein infiltration is lower in chronic rhinosinusitis with nasal polyps caused by eosinophils. There was an increase in HMGB1 expression in inflammatory cells associated with eosinophilic chronic rhinosinusitis with nasal polyps as well as non-eosinophilic chronic rhinosinusitis with nasal polyps.

DISCUSSION

It is clinically known as chronic rhinosinusitis if there is inflammation of the nasal mucosa associated with nasal polyps. According to a recently proposed hypothesis, CRS is caused by a defect or excessive immune response to foreign agents, which results in persistent influxes of inflammatory cells, since nasal anatomical variations do not seem to correlate with CRS incidence [8]. Since inflammation is primarily observed at the interface with the external environment, the incidence of CRS has not been correlated with nasal anatomical variations. It was widely believed that CRSwNP was a multifactorial disease in the past few decades [9], due to a lack of understanding of its aetiology and pathogenic mechanisms. CRS with or without nasal polyposis appears to be less determined by one microbial factor or environmental factor than by host susceptibility [10]. Cells with cilia, goblet cells, and respiratory epithelium form tight junctions to protect the sinus mucosa. Microbes and foreign substances are protected by mucociliary goblet cells. As a result of foreign proteins stimulating the immune system, the epithelial barrier is mechanically broken down, stimulating microbial

colonization. Sinonasal epithelial cells 15 are responsible for adaptive immune responses and the physical barrier. Pattern recognition receptors (PRRs) on airway epithelial cells recognize molecular patterns associated with pathogens [11]. Epithelial cells attract innate defenses by releasing chemokines, cytokines, and innate protective agents. DAMPs, as well as PAMPs, can also help cells detect cellular damage [12]. A well-established DAMP, HMGB1, causes inflammation in the lower airways. Patients with CRSwNP have greater HMGB1 protein levels in their epithelial nuclei, or in their subepithelial areas and have smaller HMGB1 proteins in their epithelial cytoplasm [13]. Chronic rhinosinusitis associated with nasal polyps containing Eos did not differ significantly in HMGB1 expression, indicating that the protein plays a role in the pathogenesis of chronic rhinosinusitis regardless of the underlying cause. Positive cells expressing IL-5, IL-8, or TNF-5 can affect the regulation of HMGB1. CRSwNP pathogenesis should be investigated through research on HMGB1.

CONCLUSIONS

This study explored the nuclear protein HMGB1 in chronic rhinosinusitis with nasal polyps (CRSwNP) and provided valuable insight into inflammatory processes within the ENT field. HMGB1 expression was found to differ in various cellular components between CRSwNP patients and controls, particularly in eosinophil infiltration. Clinical assessments, diagnostic criteria, and immunohistochemical analysis were combined in the study to understand CRSwNP pathogenesis. Eosinophilic involvement is particularly important in nasal and paranasal sinus mucosa, where HMGB1 plays a crucial role. In addition to shedlighting the link between HMGB1 and CRSwNP, the study also paves the way for future studies. We are deepening our understanding of ENT pathologies and exploring potential therapeutic targets through collaboration among several countries and universities. Continuing this research and exploring inflammatory processes in the ENT district will provide more nuanced insights that could ultimately result in better diagnosis and treatment strategies for chronic rhinosinusitis with nasal polyps. In order to advance ENT research, the authors continually discuss the results to ensure a robust and comprehensive interpretation.

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