Correlation Between Cytokine Levels in Nasal Fluid and Scored Clinical Parameters in Patients with Nasal Polyposis

Aleksandar Perić · Danilo Vojvodić ·
Aneta V. Perić · Vesna Radulović · Olivera Miljanović

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Abstract The aim of this study was to compare the cytokine levels in nasal fluid in subjects with nasal polyposis (NP) and co-morbid asthma and NP patients without asthma and to correlate these levels with clinical parameters of severity of disease. Forty NP patients (20 asthmatic and 20 nonasthmatic) were enrolled. Nasal secretion samples were collected from nasal cavities of all 40 subjects. The levels of Th1 cytokines IL-2, IL-12 and IFN- γ , Th2 cytokines IL-4, IL-5, IL-6, and IL-10, chemokine IL-8, and proinflammatory cytokines IL-1 β , TNF- α and TNF- β were measured using flow cytometric method. Each of the 40 patients was staged clinically according to global nasal symptom score, endoscopic score, and Lund-Mackay computed tomography (CT) score. The concentrations of Th2 cytokines IL-5, IL-6 and IL-10 were significantly higher ($P < 0.01$, $P < 0.01$, $P < 0.05$) in patients with NP and asthma compared with NP patients without asthma.

Positive correlations were observed between concentration of IL-2 in nasal secretions and global nasal symptom score, endoscopic score, and Lund-Mackay score only in NP patients without asthma. We also found positive correlation between CT score and the levels of IL-8, IL-4, and IL-1 β in nonasthmatic patients. Finally, our results showed a positive correlation between IL-5 levels in nasal fluid and endoscopic score only in asthmatic patients. NP in asthmatic patients have different immunological patterns compared to those without asthma. We also concluded that concentrations of cytokines measured in nasal fluid were not sensitive enough to determine the severity of disease.

Keywords Nasal polyps · Asthma · Cytokines · Nasal secretions · Flow-cytometry

Introduction

Nasal polyposis (NP) is a common chronic inflammatory disease of the nasal and paranasal sinus mucosa that has a major impact on patient's life. Polyps originate from the paranasal sinuses, most often from the anterior ethmoid complex and from there they can descend between the middle turbinate and the lateral nasal wall into the nasal cavity causing symptoms such as nasal obstruction, anosmia, sneezing, rhinorrhea, and itching [1]. Chronic persistent inflammation is a mayor factor in the development of NP [2]. Polyp tissue includes mixed inflammatory cells, of which eosinophils are the most dominant. They have the primary role in the perpetuation of chronic inflammation [2, 3]. It has been suggested that an ineffective local Th1-based immune response in these patients is associated with increased Th2-cytokine-based activity, which contributes to a chronic infection as well as to an increased presence of eosinophils,

A. Perić (✉)
Department of Otorhinolaryngology, Rhinology Unit, Military
Medical Academy, Crnotravska 17, 11040 Belgrade, Serbia
e-mail: alexneta@sezampro.rs

D. Vojvodić
Division of Clinical Immunology, Institute of Medical Research,
Military Medical Academy, Belgrade, Serbia

A. V. Perić
Institute of Pharmacy, Military Medical Academy, Belgrade,
Serbia

V. Radulović
Municipal Institute for Lung Diseases and Tuberculosis,
Belgrade, Serbia

O. Miljanović
Center of Human Genetics and Immunology,
Clinical Center of Montenegro, Podgorica, Montenegro

which then lead to further polyp formation [4]. It has been proposed that the weakened Th1 response in these patients may be secondary to the down-regulation of some specific toll-like receptors involved in the innate immune response [4].

It was found that patients with nasal polyps had asthma at an average of 29.9% in those referred to ENT departments [5]. On the other side, 7% of asthma patients had nasal polyps [2, 5]. Asthma is the most common chronic disease among children and adolescents. It is characterized by intermittent obstruction and inflammatory changes of airways and bronchial hyperresponsiveness [6]. It is known that the pathogenesis as well as pathophysiology of this disease are associated with alterations of glucocorticoid receptor function and also with persistent pulmonary inflammation, the important mediators of which are reactive oxygen and nitrogen [7].

Nasal secretions represent a first line defense medium, in which the leucocyte compartment probably acts as an efficient part of the defense mechanism along with the mucociliary transport system and the biochemical properties of the mucus [8]. To characterize inflammatory changes of the upper respiratory mucosa, cellular secretory products in nasal secretions may be determined [9]. Nasal secretions contain small amounts of cytokines, potent biologic factors involved in the regulation of inflammation and immune defense, and other inflammatory mediators expressed by various epithelial and nonepithelial cells [10]. It has been shown that nasal secretions reflects the inflammatory status of the nasal mucosa and the evolution of mucosal disease. However, it is not well known which are the mechanisms of cytokines release in nasal fluid. Results published by Ohkubo et al. [11] showed that IL-6 and IL-8 were released to the nasal fluid mainly from the migrating and epithelial cells as a result of the antigen provocation, reflex action of metacholine and by direct action of histamine. Because cytokines play a dominant role in the pathophysiology of airway disease, the cytokine profile in nasal secretions may help to recognize mechanisms underlying NP associated with bronchial asthma. The key question of this study is: can cytokine levels in nasal fluid be indicators of severity of NP?

The aim of this study was to compare the levels of these cellular secretory products in nasal secretions of asthmatic and nonasthmatic patients with nasal polyps and to correlate cytokine levels with clinical parameters of severity of NP in asthmatic and nonasthmatic patients.

Materials and Methods

Human Subjects

Forty patients (20 subjects with NP and 20 subjects with NP and concomitant asthma) were included in this

prospective study which has been performed according to the Declaration of Helsinki. Written informed consent was obtained from all subjects. The study was approved by the Ethics Committee of the Military Medical Academy, Belgrade, Serbia. There were 15 male and 5 female patients in the NP group (mean age 43.27 ± 14.72 years) and 16 male and 4 female patients in the NP with asthma group (mean age 46.52 ± 15.31 years). The diagnosis of NP was based on each patient's medical history and on the results of anterior rhinoscopy, nasal endoscopy and computed tomography. Twenty patients had diagnosis of mild persistent bronchial asthma. Diagnosis of asthma was done at the time of inclusion in the study according to the Global Initiative on Asthma (GINA) [12]. The assessment of the severity of asthma was done by a pulmonologist based on the patient's medical history, clinical data and on pulmonary function testing, including forced expiratory volume in 1 s (FEV1) and methacholine provocation test (Mch PD20). Only patients with polyps associated with mild bronchial asthma, without aspirin sensitivity were included in the study. The diagnosis of aspirin-induced asthma was done by a positive bronchial aspirin-provocation test. The other exclusion criterias were the presence of antrochoanal and sphenchoanal polyps, cystic fibrosis, and primary ciliary dyskinesia. All subjects included in this investigation did not have bronchial or respiratory tract infection and none of them were treated with oral and topical corticosteroids, antibiotics and antihistamines for at least 3 weeks before the enrollment. Skin prick test was performed on all patients for sensitivity to eighteen common allergens. A test result was considered positive when at least one of the induration diameters was 3 mm higher than that in the negative control.

Clinical Score

Only subjects with nasal symptoms whose duration was at least 2 years were included in this study. The presence of nasal symptoms associated with NP (obstruction, anosmia, sneezing, rhinorrhea, and itching) on the day of the enrollment in the study was scored according to Tsicopoulos et al. [13] from 0 to 3: zero for no symptoms, one for mild symptoms, two for moderate symptoms, and three for severe symptoms, so that the maximal global nasal symptom score was 15.

Endoscopic physical findings were scored according to Lildholdt et al. [14]. The degree of nasal polyps is classified in relation to fixed anatomical landmarks in four steps: 0 = "no polyposis", 1 = "mild polyposis (small polyps not reaching the upper edge of the inferior turbinate)", 2 = "moderate polyposis (medium sized polyps reaching between the upper and lower edges of the inferior turbinate)", 3 = "severe polyposis (large polyps reaching below

the lower edge of the inferior turbinate)". The maximal endoscopic score is six, bilaterally.

Findings on CT scans were graded according to the Lund-Mackay score [15]. The mucosal abnormalities were graded as zero (no abnormality), one (partial opacification), or two (total opacification) of the frontal, maxillary, anterior ethmoid, posterior ethmoid and sphenoid sinus, bilaterally. The ostiomeatal complexes were scored bilaterally as zero (not occluded) or two (occluded). The maximal CT grading score is 24.

Sampling of Nasal Secretions and Cytokine Determination

Nasal secretions samples were collected from nasal cavities of all 40 subjects (20 patients with NP, and 20 patients with NP and asthma) using absorption technique with cotton wool sticks, which were inserted into the nasal cavity for 60 s, as previously described [16, 17]. All of the samples were put in a 2 mL Eppendorf tube containing 1 mL of transfer media (phosphate-buffered saline with gentamicin 50 µg/mL, penicillin G 340 U/mL, fungizone 500 µg/mL) for 30 min for the cytokines to diffuse into the medium. Nasal fluid were centrifuged at 1000×g for 10 min to separate the cellular components. After centrifugation, supernatants were portioned and stored at −70°C until cytokine determination. The levels of 11 cytokines (TNF-α, TNF-β, IL-1β, IL-2, IL-4, IL-5, IL-6, IL-8, IL-10, IL-12, and IFN-γ) were measured in all 40 samples using a commercial flow cytometric kit (Flow cytomix, Bender MedSystems GmbH, Vienna, Austria) on the flow cytofluorimeter (Beckman Coulter XL-MCL, USA), which was connected with BMS Flow Cytomix Pro 2.2 Software, according to the manufacturer's instruction. The sensitivity of detection was as follows: 22 pg/mL for TNF-α; 32 pg/mL for TNF-β; 17 pg/mL for IL-1β; 28 pg/mL for IL-2; 20 pg/mL for IL-4; 30 pg/mL for IL-5; 21 pg/mL for IL-6; 13 pg/mL for IL-8; 20 pg/mL for IL-10; 5.1 pg/mL for IL-12; 8 pg/mL for IFN-γ.

Statistical Analysis

Data were expressed as mean ± standard deviation (±SD). Between-group comparisons were made by using the nonparametric Mann–Whitney *U* test. A *P* value less than 0.05 was considered statistically significant. The analysis of correlation between the parameters were evaluated by the Pearson's coefficient of correlation.

Results

Comparing two groups (NP with asthma and NP without asthma), we did not find any significant difference according

Table 1 Characteristics of study population

	Nasal polyposis	Nasal polyposis + asthma
Patients	20	20
Age	43.27 ± 14.72	46.52 ± 15.31
Male/female ratio	15/5	16/4
FEV1	100.54 ± 4.81	95.22 ± 5.35
MchPD20(µg)	1589.38 ± 57.73	548.83 ± 101.78
Allergic	10	12
Nonallergic	10	8
Nasal symptom score	10.4 ± 2.05	11.74 ± 2.78
Nasal endoscopic score	5.1 ± 1.23	6.24 ± 1.84
Lund-Mackay score	17.44 ± 3.16	18.8 ± 3.21

All results are expressed as means ± SD, FEV1 forced expiratory volume in 1 s

MchPD20(µg) is the amount of metacholine in micrograms

Table 2 Cytokine levels in nasal secretions

Parameter	NP Mean ± SD	NP with asthma Mean ± SD	<i>P</i> value
IL-12	6.28 ± 19.49	26.62 ± 43.87	<i>P</i> > 0.05
IFN-γ	37.69 ± 37.08	69.57 ± 68.83	<i>P</i> > 0.05
IL-2	229.26 ± 167.34	373.72 ± 288.63	<i>P</i> > 0.05
IL-10	31.46 ± 52.73	77.12 ± 68.24	<i>P</i> < 0.05
IL-8	169.83 ± 276.28	133.82 ± 105.61	<i>P</i> > 0.05
IL-6	56.63 ± 77.16	284.66 ± 235.64	<i>P</i> < 0.01
IL-4	577.68 ± 735.42	968.87 ± 1328.83	<i>P</i> > 0.05
IL-5	273.48 ± 678.12	635.37 ± 585.08	<i>P</i> < 0.01
IL-1β	28.27 ± 48.46	42.85 ± 43.93	<i>P</i> > 0.05
TNF-α	26.47 ± 24.57	46.45 ± 42.83	<i>P</i> > 0.05
TNF-β	166.36 ± 223.31	200.83 ± 323.53	<i>P</i> > 0.05

All results of cytokine levels were expressed in pg/mL

to the global nasal symptom score, endoscopic score and Lund-Mackay score, and presence of atopy (Table 1). We also did not find significant differences in the levels of TNF-α, TNF-β, IL-1β, IL-2, IL-4, IL-8, IL-12, and IFN-γ in the nasal secretions. The concentrations of IL-5, IL-6 and IL-10 in nasal fluid were significantly higher in patients with NP and asthma compared to patients with NP without asthma (635.37 ± 585.08 pg/mL vs. 273.48 ± 618.12 pg/mL, *P* < 0.01 for IL-5); (278.66 ± 235.64 pg/mL vs. 56.63 ± 77.16 pg/mL, *P* < 0.01 for IL-6); (77.12 ± 68.24 pg/mL vs. 31.46 ± 52.73 pg/mL, *P* < 0.05 for IL-10) (Table 2).

Positive correlations were observed between concentration of IL-2 and global nasal symptom score (*r* = 0.600; *P* = 0.018), endoscopic score (*r* = 0.544; *P* = 0.036), and Lund-Mackay score (*r* = 0.522; *P* = 0.046) only in the patients with NP without asthma. We, also found positive

correlation between Lund-Mackay score and the levels of IL-8 ($r = 0.522$; $P = 0.046$), IL-4 ($r = 0.518$; $P = 0.048$) and IL-1 β ($r = 0.548$; $P = 0.034$) in nonasthmatic patients. Finally, our results showed a positive correlation between IL-5 levels in nasal fluid and endoscopic score ($r = 0.578$; $P = 0.029$) only in asthmatic patients.

Discussion

NP may have a negative impact on lower airway biology, being involved in aggravation of bronchial disease. The mechanisms that connect upper and lower airway dysfunction are: nasal bronchial reflex, mouth breathing caused by nasal obstruction, and pulmonary aspiration of nasal contents [18, 19]. It has been shown in a rabbit model of chronic sinusitis that postnasal drainage of inflammatory mediators may affect lower airway responsiveness [18, 19]. Therefore, one can hypothesize that a local nasal inflammatory stimulus may induce a systemic effect leading to a bronchial eosinophilic inflammation [18, 19].

The initiating factor that underlie persistent inflammation and microbial colonization in NP are not well understood. Although Th2 inflammation is a central characteristic of the disease process, what triggers the local production of Th2 cytokines and infiltration of eosinophils and lymphocytes is unknown [4]. It has been shown that Th2 cytokines down-regulate toll-like receptors (TLR) expression and inhibit TLR signaling function in Th1 lymphocytes membrane. Thus, Th2 cytokines inhibit Th1 cytokines production resulting in down-regulation of antimicrobial mucosal immunity [4]. Hamilos et al. [20] found significantly higher levels of IL-5 in nasal polyp tissue from asthmatic than those from nonasthmatic subjects. The results of our research have also shown significantly higher concentration of Th2 cytokines (IL-5, IL-6 and IL-10) in nasal secretions in patients with NP and asthma than in patients without asthma. These findings underline local Th1/Th2 balance as a key factor that governs local inflammation, implying that there is a different mechanism in NP evolution in asthmatic and nonasthmatic patients.

Previous data point to IL-5 as one of the key proteins in the pathomechanism of tissue eosinophilia, enhancing the differentiation, activation, expansion, mobilisation, and insitu survival of eosinophils [21]. It is widely accepted that IL-5 plays an important role in the pathogenesis of bronchial asthma where IL-5 induces eosinophil mobilisation, B-cell growth and differentiation [21]. The main sources of IL-5 were eosinophils and mast cells [21, 22]. Preliminary findings presented by Fan et al. [22] suggest that T-cell-derived IL-5 and autosecretion of IL-5 from activated eosinophils could be the reasons for the extension and persistence of eosinophil inflammation in NP. These

findings could be an explanation why we found positive correlation between IL-5 levels in nasal fluid and endoscopic score as an indicator of spread of NP.

IL-6 is an important proinflammatory Th2 type cytokine involved in the induction of IgE synthesis as well as in mast cell proliferation and maturation [23]. IL-6 also stimulates fibroblast proliferation and collagen synthesis [23]. Immunohistochemical staining and insitu hybridization also indicated that macrophages, eosinophils, fibroblasts, and lining epithelium were the main sources of IL-6 [21, 23]. The pathogenesis of NP involves nasal polyp fibroblasts through synthesizing IL-6 to modulate the activation of immune responses (plasma cell formation) and synthesis of stroma [21, 23]. Van Zele et al. [24] showed increased colonization of NP by *Staphylococcus aureus* and presence of specific IgE directed against *Staphylococcus aureus* exotoxins in NP tissue. Rates of colonization and IgE presence in NP tissue was increased in subjects with NP and co-morbid asthma [24]. Hellings et al. [25] demonstrated that nasal application of *Staphylococcus aureus* exotoxin B in mice is capable of aggravating experimental allergic rhinitis and asthma, paralleled with an increase in bronchial and systemic Th2 cytokine levels. IL-10 is an anti-inflammatory Th2 cytokine produced by T-lymphocytes, monocytes and macrophages. It is considered to inhibit the release of IFN- γ and the cytokine synthesis of monocytes and leads to marked immunosuppression [26].

Positive correlations between IL-4, IL-1 β , and IL-8 levels in nasal secretions and Lund-Mackay score can be explained by several recently published findings. Eosinophil infiltration is regulated by numerous chemokines and adhesion molecules such as eotaxin, regulated on activation normal T-cell expressed and secreted (RANTES), and vascular cell adhesion molecule (VCAM)-1 [21, 27]. In order to infiltrate sites of inflammation, eosinophils leave the bloodstream and pass through the endothelium in four steps, namely rolling, adhesion, transendothelial migration, and chemotaxis [21]. Adhesion molecules, such as VCAM-1, play an important role during adhesion to endothelial cells [21].

Among some other Th2 cytokines, IL-4 has been shown to deliver signals that support or cause selective influx of eosinophils [21, 27]. It has been speculated that IL-4 may be involved in the induction of VCAM-1 expression on microvascular endothelium in NP [21, 27]. A study performed by Yoshifuku et al. [27] showed that IL-4 increased the secretion of eotaxin and VCAM-1 from cultured NP fibroblasts.

The strong proinflammatory cytokines IL-1 β and TNF- α can lead to increased transendothelial migration of eosinophils [21]. Results published by Saji et al. [28] suggest that nasal polyp fibroblasts play an important role in

inducing eosinophilic infiltration, through IL-1 β and TNF- α induced production of RANTES in nasal polyp fibroblasts. Invitro effects of RANTES on eosinophils include chemotaxis, transendothelial migration, induction and production of reactive oxygen, and the release of ECP [28]. IL-8 is predominantly a chemoattractant for neutrophils as well as for eosinophils after these target cells have been primed with IL-5 [21, 26].

Positive correlations between concentration of IL-2 and nasal symptom score, endoscopic score and Lund-Mackay score is difficult to explain. IL-2 is an essential growth factor for T-cells and it acts in an autocrine fashion to stimulate T-cell proliferation and also serves to regulate immunoglobulin production and the growth of cytotoxic T-lymphocytes and natural killer cells [6]. However, its role in development of NP is unclear and presents the object of further investigation.

Conclusion

Our results demonstrated that presence of Th2 cytokines, especially IL-5 and IL-6 in nasal fluid in NP patients is a prominent feature that relates to the increased inflammatory process. Our findings also suggest that upregulation of Th2 cytokines is more significant characteristic of NP in asthmatic than in nonasthmatic patients. These results showed that these patients with similar clinical findings had significantly different mediator profiles in their nasal secretions, implying clear differences in pathogenesis of their NP. The concentrations of IL-4, IL-8 and IL-1 β correlate with Lund-Mackay CT score as an indicator of severity of disease only in nonasthmatic NP patients. In the asthmatic NP patients, IL-5 levels in nasal fluid correlate with endoscopic clinical findings. Why we did not find similar correlations both in asthmatic and nonasthmatic patients? Do higher levels of these cytokines in nasal secretions may be an indicator of spread of nasal polyps? We concluded that concentrations of cytokines measured in nasal fluid were not sensitive enough to determine the severity of disease and suggest for further investigations with higher number of patients.

Conflict of interest None.

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