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Aktywność procesu zapalnego a stężenie markerów zawartych w plwocinie u dzieci z astmą oskrzelową o różnym początku choroby

Activity of the inflammatory process depending on sputum markers in children with different onset of bronchial asthma

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Streszczenie

Cel badania: Analiza aktywności procesu zapalnego w drogach oddechowych u dzieci z astmą oskrzelową w zależności od początku choroby. **Materiał i metody:** Zgodnie z zasadami bioetyki przeprowadzono retrospektywne badanie w grupie 319 dzieci chorych na astmę oskrzelową. U 257 dzieci (grupa kliniczna I) astma oskrzelowa rozwinęła się na podłożu przewlekłego obturacyjnego zapalenia oskrzeli. Druga grupa kliniczna (II) obejmowała 43 dzieci, u których astma wystąpiła po przebytym pozaszpitalnym zapaleniu płuc. Trzecią badaną grupę (III) stanowiło 19 dzieci, u których astmę rozpoznano po raz pierwszy po leczeniu stacjonarnym z powodu stanu astmatycznego. **Wyniki:** Analizując stopień nasilenia astmy oskrzelowej, stwierdzono, że u pacjentów z grupy III znamiennie częściej przebieg choroby był ciężki w porównaniu z pozostałymi pacjentami. U pacjentów z grupy I początek choroby charakteryzował się wzrostem liczby eozynofilów i niską liczbą neutrofilów w plwocinie, u dzieci z grupy II – zwiększoną liczbą eozynofilów i komórek nabłonka oraz niską liczbą limfocytów, natomiast u pacjentów z grupy III – niską liczbą eozynofilów w plwocinie przy jednoczesnym wzroście liczby neutrofilów. W szczególności statystycznie znamienny wzrost stężenia czynnika wzrostu śródbłonka naczyniowego i spadek zawartości białek kationowych, metaloproteinazy macierzy-9 oraz stężeń interleukiny 6 oraz interleukiny 13 w plwocinie wskazuje na przewagę neoangiogenezy u dzieci z grupy III. Z kolei w grupie klinicznej II procesy remodelingu były wywołane głównie procesem zapalnym z uwalnianiem wewnątrzkomórkowych białek kationowych eozynofilów. **Wnioski:** Zgromadzone dane wskazują na zróżnicowanie typu i nasilenia procesu zapalnego w obrębie dróg oddechowych w okresie dynamicznej obserwacji u dzieci zakwalifikowanych do poszczególnych porównywanych grup, co wskazuje na obecność różnic fenotypowych wynikających z odmiennego początku choroby uwarunkowanego różnymi czynnikami wyzwalającymi. Obserwowana zmienność przebiegu procesu zapalnego wskazuje, że chorzy na astmę wymagają zindywidualizowanego podejścia pozwalającego na zróżnicowaną diagnostykę i ukierunkowane leczenie przeciwzapalne uwzględniające specyfikę początku choroby.

Słowa kluczowe: astma oskrzelowa, dzieci, początek choroby, markery w plwocinie

Abstract

Aim of the study: To analyse the activity of the inflammatory process in the airways in children with bronchial asthma depending on the onset of the disease. **Materials and methods:** In compliance with the principles of bioethics, a comprehensive retrospective examination of 319 children suffering from bronchial asthma was performed. In 257 children (clinical group I), bronchial asthma developed on the background of chronic obstructive bronchitis. The second (II) clinical group included 43 children, in whom asthma occurred after community-acquired pneumonia. The third (III) clinical group consisted of 19 children in whom asthma was first verified after inpatient treatment for asthmatic status. **Results:** Based on the severity of bronchial asthma, it was found that the representatives of the clinical group III, compared with other patients, significantly more often had a severe course of the disease. For patients of the clinical group I, the onset was characterised by increased eosinophils and decreased neutrophil counts in sputum, for group II patients – increased eosinophils and epitheliocytes, but a decrease in lymphocytes, and in children of the clinical group III – low eosinophils in the sputum with a simultaneous increase in neutrophils. In particular, a statistically significant increase in the level of vascular endothelial

growth factor, and a decrease in the content of cationic proteins, matrix metalloproteinase-9, and interleukins 6 and 13, in sputum indicates the predominance of neoangiogenesis in children of the clinical group III. Instead, in the clinical group II the remodelling processes were mainly caused by the inflammatory process with the release of intracellular eosinophilic cationic proteins. **Conclusion:** These data indicate the discrete nature of the type and severity of the inflammatory process of the respiratory tract over the dynamic follow-up period in children classified in different clinical comparison groups, which suggests the presence of certain phenotypic differences due to alternative onsets of the disease, which were determined by different triggers. Such deviations in the inflammatory process indicate that patients with asthma require a personalised approach to ensure differentiated diagnostic monitoring and targeted anti-inflammatory treatment, taking into account the peculiarities of the onset of the disease.

Keywords: bronchial asthma, children, onset of the disease, sputum markers

INTRODUCTION

Physicians are always faced with the task of prescribing the necessary examinations and most effective tactics of treatment of paediatric patients, while trying to do it as non-invasively as possible. In recent years, non-invasive diagnostic procedures have been actively developed. In particular, biomarkers of the inflammatory process of the respiratory system are a very promising and attractive approach due to the need to study the characteristics to determine the type or nature of respiratory inflammation. These biomarkers are usually objectively measurable indicators of physiological or pathological processes; they are quite sensitive, reproducible and feasible in childhood[1,2].

One of the relatively non-invasive procedures for the study of cells and mediators from the lower respiratory tract is the method of sampling and analysis of induced sputum production. Sputum sampling in this case is carried out after the inhalation of nebulised hypertonic saline solution with a gradual increase in its concentrations, followed by the sampling of a certain amount of biomaterial[3,4]. The composition and number of cells in sputum can help detect various forms of airway inflammation: eosinophilic, neutrophilic, mixed, paucigranulocytic, etc.[5]. Sputum eosinophilia is usually a marker of the severity of allergic inflammation in bronchial asthma, and in such patients, as a rule, higher efficacy of basic therapy with inhaled glucocorticosteroids (IGCS) is observed[6], and at the end of the course of such therapy there is usually a decrease in bronchial hyperresponsiveness and sputum eosinophilia[7]. In these patients, in addition, there is always an increased number of epithelial cells in sputum and an elevated concentration of eosinophil cationic protein in supernatant sputum[8,9]. Another common variant of bronchial inflammation is sputum neutrophilia, which is often accompanied by a reduced number of macrophages in sputum and elevated levels of interleukin 8. Such children are less susceptible to standard basic therapy with inhaled corticosteroids[10]. However, some authors have proposed the concept of "divergent phenotypes" of asthma based on the cellular phenotypes of sputum monitoring, to classify asthma patients as poly- or paucigranulocytic phenotypes and with a predominantly cellular composition of sputum[11].

Based on the above, we considered it relevant and promising to study sputum markers in children with different onsets of bronchial asthma, and analyse the features of the content of biomarker and the nature of the inflammatory process in children with chronic (chronic obstructive bronchitis) or sudden onset (asthma status, pneumonia) of the disease.

AIM OF THE STUDY

To analyse the activity of the inflammatory process in the airways of children with bronchial asthma depending on the type of onset of the disease.

MATERIALS AND METHODS

Three hundred nineteen children were comprehensively examined in the Regional Children's Clinical Hospital in Chernivtsi by the method of "experiment-control" in parallel groups using a simple random sample. In 257 children (clinical group I) bronchial asthma developed on the background of chronic obstructive bronchitis (mean age: 11.7 ± 0.23 years, proportion of boys: 71.6%, proportion of rural residents: 55.6%). The second (II) clinical group included 43 children (average age: 9.9 ± 0.55 years, proportion of boys: 50.5%, proportion of rural residents: 72.1%), in whom asthma developed after community-acquired pneumonia. The third (III) clinical group consisted of 19 children in whom asthma was first verified after inpatient treatment for asthmatic status (average age: 7.7 ± 0.9 years, proportion of boys and rural residents: 52.6%).

The diagnosis and treatment of bronchial asthma (BA) were based on the protocol and adapted clinical guidelines approved by the Ministry of Health of Ukraine on 8 October 2013, No. 868, and recommendations included in the international harmonisation guidelines (Global Initiative for Asthma, GINA)[12] and their subsequent versions. Comprehensive laboratory and instrumental examination of patients was performed during the exacerbation and remission of the disease. The mean duration of the disease in children with bronchial asthma at the beginning of the flow-up was 4.6 ± 0.24 years.

According to the latest version of GINA, asthmatic status is defined as a qualitatively new condition that accompanies

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a long and resistant course of a severe (life-threatening) asthma attack, thus given the longitudinal nature of our follow-up, clinical group III will be conditionally marked as the one with the onset of the disease in the form of "asthmatic status."

To obtain sputum samples, a procedure was performed to induce its discharge by serial inhalation of a hypertonic solution of sodium chloride according to the method proposed by Pavord and Pizzichini[13]. The eosinophilic type of inflammation was indicated by the presence of 3.0% or more eosinophilic leukocytes in sputum. The non-eosinophilic nature of bronchitis was diagnosed based on a relative content in the cytogram of cell sediment less than 3% of eosinophils or their absolute absence. Appropriate protocols were used to study the cytological composition of sputum[14,15]. The activity of oxygen-dependent metabolism of neutrophilic and eosinophilic granulocytes in peripheral blood was assessed by the histochemical method according to spontaneous and stimulated tests with nitroblue tetrazolium staining (NBT test) using the method proposed by Park et al. The test results were evaluated by the percentage of formazan-positive cells (in %) and the histochemical index.

Determination of the following biomarkers in the supernatant of the sputum was carried out:

1. VEGF (vascular endothelial growth factor) – a three-stage "sandwich" variant of solid-phase enzyme-linked immunosorbent assay using mono- and polyclonal antibodies (reagents "VEGF-VectorBest" A-8784, RF);
2. MMP-9 (matrix metalloproteinase 9) – by the method of "sandwich" ELISA (reagents "Affymetrix eBioscience" BMS2016/2/BMS2016/2TEN (Bender MedSystems, GmbH, Austria);
3. ECP (eosinophilic cationic protein) – by ELISA (reagents "Aviscera Bioscience, INC" SK00128-01, USA);
4. interleukin 6 – three-stage "sandwich" variant of solid-phase enzyme-linked immunosorbent assay using mono- and polyclonal antibodies (reagents "VEGF-VectorBest" A-8768, RF);
5. interleukin 13 – by sandwich ELISA (reagents "Affymetrix eBioscience" BMS231/3/BMS231/3TEN (Bender MedSystems, GmbH, Austria);
6. gamma-interferon – a three-stage "sandwich" variant of solid-phase enzyme-linked immunosorbent assay using mono- and polyclonal antibodies (reagents "VEGF-VectorBest" A-8752, RF).

The results of the study were analysed from the viewpoint of biostatistics and clinical epidemiology. In the normal distribution and large sample groups, parametric methods of analysis were used, while in small samples – non-parametric methods were applied. Statistical analysis was performed using Statistica 8.0 software from StatSoft Inc. The population analysis assessed attributive (AR) and relative risk (RR), as well as the odds ratio (OR) with the calculation of confidence intervals for relative risk and odds ratio (95% confidence interval, CI).

The study was conducted in conformity with the main principles of the Helsinki Declaration on Biomedical Research, the provisions of ICH GCP (International Conference of Harmonisation – Good Clinical Practice), and the order of the Ministry of Health of Ukraine (No. 690, 23 September 2009), as amended by the Order of the Ministry of Health of Ukraine (No. 523, 12 July 2012), in compliance with the ethical principles and recommendations involving people as subjects set out in the Belmont Report. The design of the study was based on adherence to the principles of confidentiality and respect for the child as a person incapable of self-defence, the concept of informed consent, taking into account the benefits over the risk of harm, and other ethical principles applicable to research subjects. The protocol for the examination of children, the scope of examination, and the map of informed consent were approved by the Bioethics Committee at the Regional Children's Clinical Hospital Chernivtsi and the Ethics Committee of the Bukovinian State Medical University (BSMU) (Minutes No. 3, 21 November 2019). The study was confined to patients in the Chernivtsi region, and the accuracy of the laboratory data was limited by the methods of determination.

RESULTS

The allergic form of asthma was confirmed in 56.0% of patients in group I, in 32.6% of cases in group II, and 57.9% of patients in group III (p I, III: II <0.05), while the mixed form was found in 44.0%, 67.4%, and 42.1% of children, respectively (p I, III: II <0.05). However, the onset of the disease in children under three years of age (phenotype of early-onset asthma) was significantly more common in group III patients, and after 6 years (phenotype of late-onset asthma) – in patients classified in the clinical groups I and II.

According to the severity of bronchial asthma, it was found that the representatives of the clinical group III, compared with other patients, had a severe course of the disease significantly more often, and the ratio of the chances of severe asthma in the future in these children compared with the cohort group I was 6.8 (95% CI: 3.59–12.81), relative risk 2.4, attributive risk 44.2%, with a plausibility ratio of 3.1.

To assess the nature of bronchitis, a cytomorphological analysis of the cellular composition of induced sputum in children from the compared groups was conducted (Tab. 1). For patients in clinical group I, the onset was characterised by increased eosinophil and decreased neutrophil counts in sputum, for patients in group II – increased eosinophils and epitheliocytes, but a decrease in lymphocytes, and for children in clinical group III – low eosinophils in sputum with a simultaneous increase in neutrophils.

More active stimulation of neutrophils in patients classified in clinical group III was confirmed by the results of the NBT test of sputum neutrophils in children in the compared groups (Tab. 2).

According to the acquired data, the main feature of the local inflammatory process in the bronchi in patients with

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Patient group	Cellular composition, %				
	Eosinophils	Neutrophils	Lymphocytes	Macrophages	Epitheliocytes
Reference values	0.8 ± 0.06	56.0 ± 2.96	4.8 ± 0.89	38.3 ± 3.95	19.7 ± 5.44
At the beginning of follow-up					
Group I	7.5 ± 1.14	53.4 ± 2.12	8.7 ± 0.90	23.9 ± 1.85	35.0 ± 1.99
Group II	8.2 ± 1.18	60.9 ± 4.27	6.7 ± 1.55	22.8 ± 3.02	41.5 ± 5.16
Group III	3.1 ± 1.25	68.2 ± 5.66	9.1 ± 3.30	17.1 ± 2.92	35.9 ± 2.31
p	I, II : III <0.05	I : III <0.05	>0.05	I : III <0.05	>0.05
At the end of follow-up					
Group I	10.1 ± 2.62	55.3 ± 4.08	8.2 ± 1.58	18.4 ± 3.13	27.3 ± 3.55
Group II	9.0 ± 3.44	58.9 ± 8.69	4.5 ± 1.72	10.5 ± 2.54	27.8 ± 6.07
Group III	2.0 ± 0.89	51.8 ± 7.11	4.2 ± 1.69	6.8 ± 1.08	22.4 ± 10.53
p	I, II : III <0.05	>0.05	>0.05	I : III <0.05	>0.05
* *p* – difference reliability by Student's criteria.					

Tab. 1. Cellular composition of sputum in patients with bronchial asthma during the dynamic follow-up period (M ± m)

Clinical groups	NBT test of sputum neutrophils		Neutrophil stimulation index	Neutrophil reserve
	Spontaneous	Stimulated		
Group I	16.7 ± 1.12	18.4 ± 1.22	1.1 ± 0.11	2.0 ± 0.74
Group II	18.2 ± 2.40	20.1 ± 2.97	1.2 ± 0.09	2.4 ± 1.45
Group III	11.0 ± 2.00	17.5 ± 3.50	1.6 ± 0.01	6.5 ± 1.50
p	I, II : III <0.05	>0.05	I, II : III <0.05	I, II : III <0.05
* *p* – difference reliability by Student's criteria.				

Tab. 2. Indicators of the test with nitroblue tetrazolium staining (NBT test) of sputum neutrophils in children from compared groups (in % of formazan-positive cells) (M ± m)

Clinical groups	VEGF, pg/mL	ECP, ng/mL	MMP-9, ng/mL	IL-6, pg/mL	IL-13, pg/mL
Group I	123.9 ± 10.69	2.2 ± 0.29	5.7 ± 0.47	8.1 ± 0.74	29.4 ± 4.39
Group II	110.4 ± 6.49	2.4 ± 0.56	4.5 ± 1.20	9.0 ± 2.42	40.3 ± 11.05
Group III	174.5 ± 25.50	0.6 ± 0.06	1.8 ± 0.70	4.4 ± 0.60	3.3 ± 0.25
p	I, II : III <0.05	I, II : III <0.05	I, II : III <0.05	I, II : III <0.05	I, II : III <0.05
* *p* – difference reliability by Student's criteria. **ECP** – eosinophilic cationic protein; **IL** – interleukin; **MMP-9** – matrix metalloproteinase-9; **VEGF** – vascular endothelial growth factor.					

Tab. 3. Sputum biomarkers in children from compared groups (M ± m)

asthma developing in connection with asthmatic status was a decrease in the content of formazan-positive cells in spontaneous and stimulated NBT test with activation of oxygen-dependent metabolism of neutrophilic granulocytes in sputum with significantly higher indicators of the stimulation index and the reserve of their microbicidality, which can then be used as a non-invasive prognostic marker of the severity of persistence of asthma.

DISCUSSION

Many researchers point out the need for more personalised and targeted therapy and diagnosis of asthma in children[16]. Furthermore, according to a recent study[17], the process of persistent allergic inflammation in the bronchi is superseded by morphological changes in the form of remodelling and insensitivity to bronchodilator drugs. Based on the above considerations, we believe it relevant to analyse the biomarkers for the remodelling in patients' sputum, to optimise the assessment of efficacy of treatment and the prognosis of BA for children with different types of onset of the disease. Based on the literature data on possible markers of such irreversible changes in the bronchi[18,19], we studied the content of endothelial vascular growth factor (VEGF), eosinophilic cationic protein (ECP), cationic proteins of eosinophils, matrix metalloproteinase (MMP-9) and interleukins 6, 13 in the supernatant of sputum of the examined children (Tab. 3).

The results indicate that in patients with pneumonia in the onset of asthma, the local inflammatory process of the bronchi was characterised by a hypergranulocytic inflammatory phenotype with the most pronounced desquamative processes of the respiratory epithelium. Patients in whom asthma developed from asthmatic status were characterised by a predominance of the neutrophil variant of inflammation with lymphocytic infiltration and a decrease in the pool of macrophages, indicating impairment of the protective function of these cells[16]. Based on the data obtained,

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it can be argued that markers of the cellular composition of sputum in asthma patients can be used for diagnostic and prognostic purposes, in the former case – to exclude concomitant pneumonia, and in the latter – to verify deep inflammatory processes in the airways with their remodelling.
In the process of dynamic follow-up, it was found that a basic anti-inflammatory treatment was accompanied by consistent changes in the cellular composition of sputum in patients of clinical comparison groups. In particular, a significant reduction in desquamative-inflammatory processes in the respiratory system was achieved, as evidenced by a decrease in the percentage of epitheliocytes in the mucosa of patients in group I by 1.3 times, patients in group II – by 1.5 times, and in children with asthma developing from asthmatic status – 1.6 times. This was accompanied by a decrease in the activity of macrophages, the severity of which was the highest in representatives of the second clinical group (2.2-fold decrease) and patients of the third group (2.5-fold decrease) as well as a decrease in lymphocyte content by 1.5 and 2.2 times.
It should be noted that no fundamental changes of the type of the inflammatory process of the bronchi occurred. It remained mainly eosinophilic in the children of groups I and II, and neutrophilic with a statistically significant decrease in the content of these cells in sputum ($p = 0.05$) in children of clinical group III. The established features, on the one hand, reflect the efficacy of treatment, and on the other – reveal the phenotypic features of the nature of the inflammatory process in the bronchi in children with an alternative onset of asthma.
As follows from the presented data, in children with different types of onset of asthma, one can assume the presence of a multivariate nature of airway remodelling. In particular, a statistically significant increase in the content of VEGF, a decrease in the content of cationic proteins, MMP-9, and interleukins 6, and 13 in sputum indicates the predominance of neoangiogenesis in children of clinical group III. Instead, among the representatives of the clinical group II the remodelling processes were mainly caused by the inflammatory process with the release of intracellular eosinophilic cationic proteins. At the same time, in patients in whom asthma developed as a result of repeated episodes of obstructive bronchitis, airway remodelling was caused by structural rearrangements of the epithelial-mesenchymal unit with the accumulation of matrix metalloproteinase-9 in sputum.
The paper showed that the risk ratio of bronchial remodelling with VEGF content in sputum was more than 170 pg/mL in children of clinical group III relative to patients of group I was 2.2, the relative risk was 1.0 with a likelihood ratio of 1.4. The ratio of the chances of maintaining an intense inflammatory process in the airways with their subsequent remodelling in patients of clinical group II relative to patients of group I with a cationic protein content of more than 2.0 ng/mL and an interleukin 6 content of more than 9.0 pg/mL reached 3.8 (95% CI: 1.02–13.80), relative risk 1.4, attributive risk 25.6%. It was found that the content of such a biomarker as gamma-interferon, although not significant, also varied in the supernatant of sputum of children with asthma. Thus, in patients included in clinical group III, its concentration was higher (52.5 ± 3.25 ng/mL) compared with representatives of group I (32.1 ± 4.23 ng/mL) and group II (30.4 ± 7.56 ng/mL), $p > 0.05$.
Therefore, the identified changes may indicate the presence of phenotypic heterogeneity of the chronic inflammatory process in the airways of children with an alternative onset of asthma, which, in turn, justifies the development of a personalised approach to basic anti-inflammatory treatment.

CONCLUSIONS

1. The study data indicate the discrete nature of the type and severity of the inflammatory process in the respiratory system in the dynamic follow-up of children in the clinical comparison groups, which suggests the presence of certain phenotypic differences due to the alternative onset of the disease, which in turn was determined by different triggers.
2. It was found that recurrent obstructive bronchitis in the onset of asthma was associated with the eosinophilic nature of the inflammatory process, and the accumulation of MMP-9 as a marker of bronchial remodelling in the mucosa of sputum. The pneumonia process in the onset of bronchial asthma was accompanied by the hypergranulocytic character of the inflammatory response. The phenotype of asthma which develops from the clinical manifestation of asthmatic status is associated with the neutrophilic nature of the inflammatory process, with activation of the oxygen-dependent metabolism of sputum granulocytes causing a 2.2-fold increase in the concentration of endothelial vascular growth factor in sputum.
3. Such variation in the inflammatory process indicates that patients with asthma require a personalised approach involving differentiated diagnostic monitoring and targeted anti-inflammatory treatment, taking into account the peculiarities of the onset of the disease.

Conflict of interest

The authors do not report any financial or personal connections with other persons or organisations which might negatively affect the contents of this publication and/or claim authorship rights to this publication.

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