

Expression of the apoptosis inhibitor Bcl-2 in sputum eosinophils from children with acute asthma

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Summary

Background Apoptosis of eosinophils is of increasingly important value in modulating allergic airway inflammation in asthma. Our purpose was to investigate the degree of expression of the antiapoptotic B-cell lymphoma/leukaemia-2 (Bcl-2) protein in sputum eosinophils during acute asthma exacerbation and its relationship with exacerbation severity.

Methods Sputum was obtained from 33 asthmatic children and 15 healthy children as a control group. Patients were studied during an acute asthma exacerbation. They were classified according to the severity of exacerbation into mild, moderate and severe ($n = 11$ for each). Patients with severe exacerbation were followed up until remission and another sputum sample was obtained. Number of sputum eosinophils was expressed as percentage of leucocytes. Bcl-2 expression in sputum eosinophils was assessed by immunohistochemical staining techniques; the results were expressed as percentage of positively stained cells over total eosinophils.

Results Sputum eosinophils and Bcl-2⁺ eosinophils' percentages were significantly higher in patients with acute exacerbation than controls ($P < 0.01$). Patients with severe exacerbation had significantly higher sputum Bcl-2⁺ eosinophils' percentage than those with mild-to-moderate exacerbation (mean $\pm$ SD = $42.4 \pm 31.96\%$ vs. $5.7 \pm 14.5\%$, $P < 0.01$). A significant negative correlation was found between Bcl-2⁺ eosinophils' percentage and peak expiratory flow rate % predicted ($P < 0.05$). After remission, patients with severe exacerbation showed a significant decrease of Bcl-2⁺ eosinophils' percentage ($P < 0.05$).

Conclusion Our findings suggest that Bcl-2 prolongs survival and decreases apoptosis of airway eosinophils in asthma especially during exacerbation. Eosinophil apoptosis and Bcl-2 represent a target for new and effective therapeutic strategies of asthma.

Keywords apoptosis, asthma, Bcl-2, eosinophils, sputum

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Introduction

Eosinophils play a major role in the onset and maintenance of bronchial inflammation and tissue injury in asthma [1, 2].

The prolongation of eosinophil survival is important in the pathogenesis of asthmatic airway inflammation. Apoptosis (or programmed cell death) is thought to be critically important in promoting the clearance of inflammatory cells and the resolution of inflammation. Apoptosis of eosinophils may be clinically relevant in asthma, promoting the removal of airway eosinophils and contributing to clinical improvement [3, 4].

Apoptosis is morphologically characterized by chromatin condensation, genomic DNA fragmentation and cell membrane dissociation. The genetic regulation of apoptosis involves a large number of genes classified into three categories: genes suppressing apoptosis, such as those belonging to the Bcl-2 (B-cell lymphoma/leukaemia-2) family

[5]; genes acting as effectors of apoptosis, such as caspases [6]; and intermediate genes upstream, such as Fas/Fas ligand, p53 and myc [7, 8].

The Bcl-2 gene was first discovered because of its involvement in B-cell malignancies, where t(14;18) chromosomal translocations activate the gene in about 80–90% of follicular non-Hodgkin's lymphomas [9]. Bcl-2 protein is an intracellular protein that inhibits apoptotic death induced by a variety of stimuli in different cell types [10].

Vignola et al. [11] reported that Bcl-2⁺ cells in bronchial biopsy specimens from patients with asthma were higher than those from control subjects and from subjects with chronic bronchitis; the number of Bcl-2⁺ cells correlated with the severity of the disease.

This study was undertaken to determine the degree of expression of the antiapoptotic Bcl-2 protein in sputum eosinophils of children with acute asthma and its relationship with the severity of the exacerbation. We also wanted to explore whether the degree of Bcl-2 expression in sputum eosinophils in patients with severe asthma exacerbation changes when they are followed up till their steady state (remission).

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Materials and methods

Subjects

This study was conducted on 33 asthmatic children recruited from the Pediatric Allergy and Immunology Unit, emergency room and outpatient clinics of Ain Shams University Children's Hospital, Cairo, Egypt, over a period of 4 months.

They were 19(58%) males and 14(42%) females; their ages ranged between 5 and 15 years with a mean of 9.53 ± 3.12 years.

It also included 15 healthy children without a history of asthma or other atopic conditions and without a family history of atopy as a control group. They were nine (60%) males and six (40%) females; their ages ranged between 7 and 14 years with a mean of 10.73 ± 2.19 years.

The diagnosis of asthma was based on the clinical symptoms and signs of episodic wheezing, chest tightness and dyspnoea that improve at least partially after bronchodilator therapy.

Patients were excluded from the study if they had clinical evidence of respiratory infection. None of the patients had chest radiograph or sputum culture evidence of pulmonary bacterial infection during the study period.

Ain Shams University Children's Hospital Ethics Committee approved the study. Informed consent was obtained from patients or caregivers of each patient or control subject before enrolment in the study.

Study design

Patients were studied during an acute asthma exacerbation (defined by increasing cough, wheezing, dyspnoea and nocturnal symptoms).

The severity of acute asthma exacerbations was classified by symptoms (breathlessness, talking and alertness), signs (respiratory rate, use of accessory muscles, wheezes, pulse/min and pulsus paradoxus) and functional assessment (peak expiratory flow rate (PEFR), arterial blood gas analysis) following the National Heart, Lung and Blood Institute, Expert Panel Report 2, 1997 [12].

Patients were classified according to the severity of acute asthma exacerbation into 3 groups; mild, moderate and severe. Each group comprised 11 patients.

The patients with severe asthma exacerbation were followed up for 2–3 weeks until stabilization of their condition and quiescence of asthma symptoms by treatment (i.e. remission or steady state); then, follow-up blood and sputum samples were collected.

Methods

Measurement of peak expiratory flow rate Mini-Wright Peak Flow Meter (Clement Clarke International Ltd, Harlow, England) was used.

Since the parents did not know their children's personal best, the estimation of the expected PEFR was obtained from a standardized chart that approximates PEFR by the child's height [13].

The PEFR of each patient was compared with the normal population and % predicted was calculated.

Complete blood count Venous blood sample from each subject was used for measurement of total leucocytic count and blood eosinophils. One mL of peripheral blood was collected into sterile EDTA tubes and assayed by Coulter counter on the same day (Coulter MicroDiff 18, Coulter Corp., Miami, FL, USA). The differential leucocytic counts were estimated manually from the blood film and expressed in absolute count values.

Immunoglobulin E assay Serum total IgE was assayed in all subjects by ELISA (Eurogenetics, IgE Quantitative, Tessen derlo, Belgium). Results are expressed in IU/mL. Owing to the variability in serum total IgE levels with age in childhood, we calculated the percentage values from the reference ranges [14] by dividing the subject's level by the highest normal for age $\times 100$. IgE levels used in the correlations were both the measured and the calculated percentage values.

Sputum induction Subjects under study were selected above 5 years of age so that adequate sputum samples could be obtained spontaneously when required. Sputum was induced only when it could not be produced spontaneously. The sputum induction was performed as a modification of the method described by Fahy et al. [15]. All subjects were premedicated with two puffs of inhaled salbutamol (200 μ g) 30 min before sputum induction. To minimize contamination with saliva and postnasal drip, subjects were asked to rinse their mouths and blow their noses before induction and, whenever possible, before expectoration. Subjects inhaled 3% hypertonic saline solution aerosols generated by Farnasol compressor nebulizer (CA-MI s.n.c., Pilastro (PR), Italy).

Hypertonic saline was inhaled for 10–15 min according to the severity of asthma until an adequate volume of sputum was expectorated. Patients were asked to cough deeply and frequently during hypertonic saline inhalation. They were asked to cough the sputum into a sterile plastic container. The sample volumes and duration of sputum induction were recorded. Sputum induction was stopped if the PEFR % predicted fell by $\geq 15\%$.

Sputum processing Sputum was selected from saliva and processed within 2 h. The method of sputum examination described by Popov et al. [16] was modified [17]. Sputum obtained was treated by adding equal volumes of 0.1% of dithiothreitol (ICN Biomedicals, Inc., Irvine, CA, USA), followed by equal volumes of Dulbecco's phosphate-buffered saline (D-PBS). The sample was then mixed gently and placed in shaking water bath at 37°C for 15 min to ensure complete homogenization. The sample was removed from the water bath periodically for further brief gentle mixing. Then, centrifugation was carried out at $1500 \times g$ for 10 min. The cell pellet was spread on three slides. Two were air dried for 10 min, fixed in cold 0.25% formalin for 5 min and stored at -70°C for immunohistochemical analysis. The third slide was stained with Leishman and a differential cell count of 400 non-squamous cells was performed. Eosinophils in sputum are expressed as percentage of leucocytes. Evaluation was carried out by a single person, blind to the clinical conditions of the patients.

Immunohistochemical analysis of Bcl-2 Immunohistochemical analysis was performed on sputum slides using

monoclonal mouse anti-human Bcl-2 oncoprotein as the primary antibody (DAKO Corporation, Carpinteria, CA, USA). The immunoenzymatic staining was performed using the universal DAKO labelled streptavidin-biotin 2 system, horseradish peroxidase (DAKO LSAB 2 system, HRP). Following the manufacturer's instructions, the 5–10 min incubation with 3-3' diaminobenzidine substrate–chromogen solution resulted in a brown-coloured precipitate at the antigen site.

All slides were examined using standard light microscopy and scored as percentage of positive eosinophils over total eosinophils. Eosinophils with positive immunostaining with Bcl-2 were distinguished by their morphological characteristics. The intensity of the staining reaction was not quantified. The results were expressed as percentage of positively stained cells over total eosinophils.

Statistical analysis

The results were analysed by commercially available software package (Stat View, Abacus Concepts, Inc., Berkley, CA, USA). The data are presented as mean and standard deviation (SD). Student's *t*-test was used to compare between two groups as regards parametric data (age, weight, height and PEFr), whereas the Mann–Whitney *U*-test was used for non-parametric data (age of onset, asthma duration, blood eosinophils count, IgE, sputum eosinophils' percentage and sputum Bcl-2⁺ eosinophils' percentage). Paired *t*-test and Wilcoxon signed rank test (for non-parametric data) were used to compare between the same group in two repeated measurements as in patients with severe acute asthma exacerbation vs. remission state. Correlation between variables was determined by Spearman's rank correlation coefficient. After analysis, a probability *P*-value <0.05 was considered significant.

Results

The characteristics of the study subjects are shown in Table 1. Blood eosinophils were significantly higher in patients with severe than those with moderate asthma exacerbation, and higher in patients with moderate than those with mild asthma exacerbation.

Proportion of eosinophils in sputum

Patients with acute asthma exacerbation compiled as one group had higher sputum eosinophils' percentage than control group (mean $\pm$ SD = $2.2 \pm 2.4\%$ vs. $0.2\% \pm 0.6\%$, respectively; $z = 3.2$, $P < 0.01$). Sputum eosinophils' percentage was higher in case of severe than moderate and in case of moderate than mild exacerbation, however the difference did not reach statistical significance ($2.6 \pm 2.9\%$, $2.1 \pm 2.7\%$, $1.9 \pm 1.6\%$ in severe, moderate and mild exacerbation respectively; $P > 0.05$).

Sputum Bcl-2⁺ eosinophils

Percentage of Bcl-2⁺ eosinophils in sputum was significantly higher in patients with acute asthma exacerbation (compiled as one group) than controls (mean $\pm$ SD: $17.9 \pm 27.7\%$ vs. $0.0 \pm 0.0\%$; $P < 0.01$). Patients with mild-to-moderate asthma exacerbation, compiled together ($n = 22$), had significantly lower percentage of sputum Bcl-2⁺ eosinophils than those with severe exacerbation ($n = 11$) (mean $\pm$ SD: $5.7 \pm 14.5\%$ vs. $42.4 \pm 31.9\%$; $z = 2.6$, $P < 0.01$).

Comparison of the sputum Bcl-2⁺ eosinophils among patients with mild ($1.45 \pm 2.81\%$), moderate ($9.91 \pm 19.88\%$) and severe exacerbation ($42.36 \pm 31.96\%$) as separate groups revealed lower values in mild ($n = 11$) and moderate ($n = 11$) than severe ($n = 11$) exacerbation ($P < 0.05$ for both) and comparable values in mild and moderate exacerbation ($P > 0.05$) (Fig. 1).

Patients with severe acute asthma exacerbation followed till remission

When patients with severe acute asthma exacerbation were followed till remission there was a significant increase in PEFr ($36.6 \pm 7.8\%$ vs. $79.2 \pm 8.4\%$ predicted, $P < 0.01$), a significant decrease in blood eosinophils (1532.5 ± 649.7 vs. 278.1 ± 196.2 cell/mm³, $P < 0.01$) and a decrease in percentage of sputum Bcl-2⁺ eosinophils ($42.4 \pm 32.0\%$ vs. $3.9 \pm 8.0\%$, $P < 0.05$) (Fig. 2). There was a decrease in sputum eosinophils' percentage ($2.6 \pm 2.9\%$ vs. $1.2 \pm 1.6\%$, $P > 0.05$) but this decrease did not reach statistical significance.

Patients with severe asthma exacerbation during remission state still had higher blood eosinophils, sputum eosinophils' percentage and sputum Bcl-2⁺ eosinophils' percentage than

Table 1. Characteristics of asthmatic patients and controls

Variable	Mild exacerbation ($n = 11$, mean $\pm$ SD)	Moderate exacerbation ($n = 11$, mean $\pm$ SD)	Severe exacerbation ($n = 11$, mean $\pm$ SD)	Controls ($n = 15$, mean $\pm$ SD)	<i>P</i>		
					Mild vs. moderate	Mild vs. severe	Moderate vs. severe
Age (years)	9.18 ± 3.06	9.05 ± 3.55	10.36 ± 2.84	10.73 ± 2.19	NS	NS	NS
Weight (kg)	30.45 ± 13.09	30.86 ± 11.18	33.55 ± 12.28	32.93 ± 6.12	NS	NS	NS
Height (cm)	127.91 ± 14.65	128.18 ± 19.35	130.00 ± 11.97	130.80 ± 6.18	NS	NS	NS
Age of onset (months)	20.27 ± 29.32	25.09 ± 30.43	36.45 ± 54.57		NS	NS	NS
Asthma duration (years)	7.55 ± 3.34	6.91 ± 3.40	8.59 ± 3.71		NS	NS	NS
PEFR (% predicted)	86.00 ± 4.12	75.00 ± 5.74	36.55 ± 7.78		<0.0001	<0.0001	<0.0001
BL Eos (cells/mm ³)	205.55 ± 187.19	545.36 ± 493.52	1532.45 ± 649.69	113.20 ± 66.23	<0.05	<0.0001	<0.01
Sp. Eos (%)	1.91 ± 1.58	2.09 ± 2.70	2.55 ± 2.94	0.20 ± 0.56	NS	NS	NS
IgE (IU/mL)	218.30 ± 292.69	216.50 ± 156.54	304.00 ± 325.44	19.50 ± 55.32	NS	NS	NS

PEFR, peak expiratory flow rate; BL Eos, blood eosinophils; Sp. Eos, sputum eosinophils; NS, non-significant.

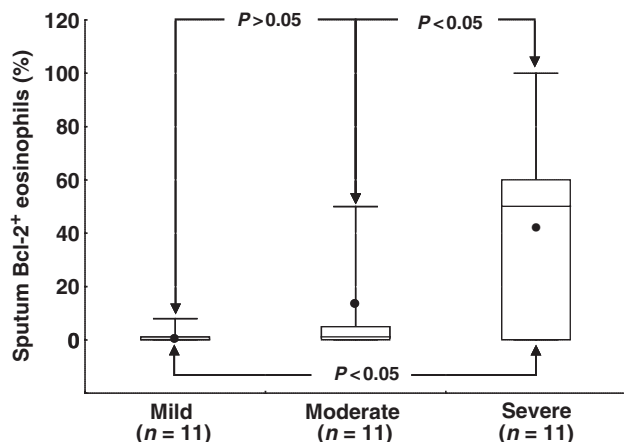


Fig. 1. Comparison of sputum Bcl-2⁺ eosinophils' percentage between patients with mild, moderate and severe asthma exacerbation. Horizontal lines indicate median values and boxes enclose interquartiles (25th and 75th percentiles). Ranges are marked as maximum and minimum, and dots represent mean values. Mann–Whitney *U*-test is used for the comparison.

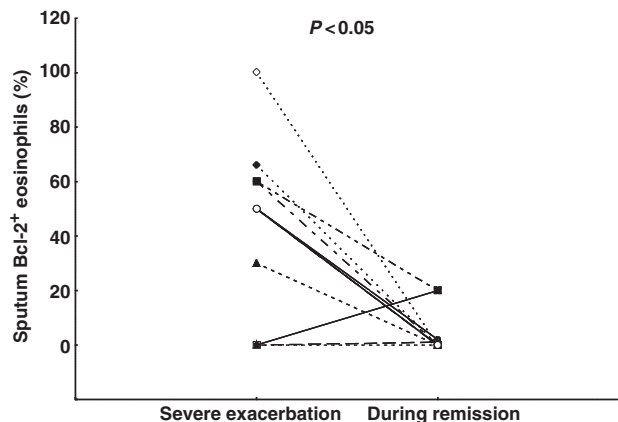


Fig. 2. Comparison of sputum Bcl-2⁺ eosinophils' percentage between exacerbation and remission in patients with severe acute asthma exacerbation.

controls; however, the difference was statistically significant only in case of blood eosinophils ($P < 0.05$).

Correlation between Bcl-2⁺ eosinophils' percentage and clinical and laboratory findings

There was a significant negative correlation between sputum Bcl-2⁺ eosinophils' percentage and PEFR ($r = -0.37$, $P < 0.05$), as seen in Fig. 3. On the other hand, there was a positive correlation between sputum Bcl-2⁺ eosinophils' percentage and eosinophils count in blood ($r = 0.46$, $P < 0.05$). Sputum Bcl-2⁺ eosinophils' percentage did not correlate to IgE levels ($r = 0.32$, $P > 0.05$).

There was a positive correlation between sputum Bcl-2⁺ eosinophils' percentage and sputum eosinophils' percentage ($r = 0.582$, $P < 0.05$).

Discussion

Eosinophils are known to play a pivotal role in asthmatic airway inflammation [18]. It has been suggested that a defect

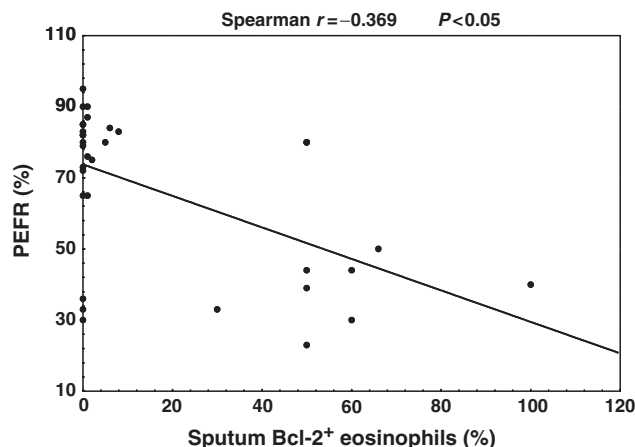


Fig. 3. Correlation between sputum Bcl-2⁺ eosinophils' percentage and peak expiratory flow rate (PEFR) % predicted.

in eosinophil apoptosis would lead to the development and persistence of allergic airway inflammation in asthma [2]. Bcl-2 prevents apoptosis either through altering cell cycle rate or by activating antioxidant-associated mechanisms [3]. It has been reported that Bcl-2⁺ cells are increased in bronchial biopsy specimens from patients with asthma [11].

The present study demonstrated higher levels of sputum Bcl-2⁺ eosinophils in patients with acute asthma exacerbation than controls and Bcl-2⁺ eosinophils' percentage was significantly higher in patients with severe than those with mild and moderate exacerbation. This was in agreement with Jang et al. [19], who used immunocytochemical staining to detect apoptosis proteins. They found that Bcl-2⁺ eosinophils were significantly more numerous in the cytoplasm of sputum eosinophils from patients with severe to life threatening asthma exacerbation than in those with mild-to-moderate asthma exacerbation. Also, Duncan et al. [20] demonstrated a relationship between reduced sputum eosinophil apoptosis and increased clinical severity of chronic stable asthma. They found that mild asthmatic subjects had lower percentage sputum eosinophils and a higher eosinophil apoptotic ratio than moderate or chronic severe asthmatics.

The present study revealed a significant negative correlation between Bcl-2⁺ eosinophils and PEFR (an indicator of the severity of asthma exacerbation). Similarly, Jang et al. [19] reported a significant negative correlation between Bcl-2⁺ eosinophils and FEV₁ (forced expiratory volume in 1 s)/forced vital capacity. They also found a significant positive correlation between sputum Bcl-2⁺ eosinophils and eosinophil activation markers such as IL-5 and eosinophil cationic protein (ECP) levels in sputum supernatant. Taken together, expression of Bcl-2 in the sputum eosinophils is a marker of severity of airway inflammation and clinical severity. Jang et al. [19] in their study found that IL-5 was frequently detected in patients with severe to life threatening asthma and expression of Bcl-2 protein in sputum eosinophils was related to the severity of asthma, suggesting that IL-5 prolongs the survival of eosinophils. Moreover, *in vitro* studies revealed that culturing eosinophils in the presence of IL-5 reduced apoptosis and induced Bcl-2 mRNA and protein expression [21]. Thus, IL-5 has an inhibitory effect on eosinophil apoptosis by regulating Bcl-2 expression [22].

Vignola et al. [11] studied apoptosis in eosinophils, macrophages and T lymphocytes in bronchial biopsy specimens obtained from asthmatic subjects and subjects with chronic bronchitis. In asthma, Bcl-2⁺ cells were higher than in control subjects and subjects with chronic bronchitis, and they were significantly correlated with the severity of asthma. Hamzaoui et al. [23] focused on sputum lymphocytes, whereas they found that the percentage of intensity of the proto-oncogene Bcl-2 was increased more in patients with asthma when compared with the healthy control group.

This study demonstrated that the percentage of sputum eosinophils was significantly higher in patients with acute asthma exacerbation than controls, similar to what was reported by previous investigators [24–26]. Eosinophilia, measured both in the peripheral circulation and in the airways, is a characteristic feature of asthma. Furthermore, the severity of the eosinophilia is correlated with objective markers of the severity of asthma such as lung function [27, 28].

Woolley et al. [29] examined eosinophils in sputum samples during an asthma exacerbation and two weeks after corticosteroid treatment of the exacerbation. They found improvement of airway inflammation and subsidence of eosinophilic inflammation, with a significant decrease in the number of airway eosinophils and the percentage of activated eosinophils and an increase in the proportion of apoptotic airway eosinophils. Similarly, our results revealed that when patients with severe asthma exacerbation were followed till remission, blood eosinophils and sputum Bcl-2⁺ eosinophils' percentage decreased significantly, whereas sputum eosinophils' percentage decreased but this decrease did not reach statistical significance. This indicates that the decrease in Bcl-2 expression is associated with improvement of acute asthma exacerbation, and apoptosis may represent a mechanism that promotes the resolution of eosinophilic inflammation in asthma. In our study, patients with severe asthma exacerbation during remission had higher blood eosinophils, sputum eosinophils' percentage than controls; however, the difference was statistically significant only in case of blood eosinophils. This indicates that airway inflammation is still present during remission but to a less extent than during exacerbation.

In our study, there was a significant positive correlation between sputum Bcl-2⁺ eosinophils' percentage and sputum eosinophils' percentage similar to what has been previously reported [19]. This demonstrates the role of Bcl-2 in increasing eosinophil infiltration in airways.

In summary, there is increased expression of Bcl-2 in eosinophils from sputum of subjects with acute asthma exacerbation than controls, which is higher in patients with severe than those with mild-to-moderate asthma exacerbation. Sputum Bcl-2⁺ eosinophils decreased when patients with severe asthma exacerbation were followed in remission.

In conclusion, our results suggest that Bcl-2 may prolong survival and decrease apoptosis of airway eosinophils in asthma especially during exacerbation, thus contributing to asthmatic airway inflammation.

Further studies are required to study the effect of other apoptosis inhibitors or inducers such as p53, Fas-ligand, Bax, Bcl-xs and others not only on eosinophils but also on other inflammatory cells such as lymphocytes and macrophages. These studies could identify potential new targets for the treatment of asthma and other allergic diseases.

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