Levels of Interleukin 17, Fas and Eosinophil Apoptosis in Bronchial Asthma Patients

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ABSTRACT

Bronchial asthma is a chronic inflammatory disorder of the airway in which many cells and cellular elements play a role. The chronic inflammation causes an associated increase in airway hyperresponsiveness that leads to recurrent episodes of wheezing, breathlessness, chest tightness and coughing particularly at night or in the early morning. These episodes are usually associated with widespread but variable airway obstruction that is often reversible either spontaneously or with treatment. IL-17 family members belong to a distinct category of cytokines that coordinate local tissue inflammation by inducing the release of pro-inflammatory and neutrophil-mobilizing cytokines. The importance of the IL-17 family in inflammatory and autoimmune disease is becoming increasingly apparent. In this prospective study, we measured sputum IL-17 and serum sFAS in bronchial asthma patients of various disease severity by ELISA technique and also detected sputum eosinophils apoptotic ratio (AR). This study was carried out at Mansoura University Hospitals, Medical Microbiology & Immunology and Thoracic Medicine Departments from August 2007 to August 2009. Fifty bronchial asthma patients and twenty healthy non-smoker subjects were enrolled in the study after informed consent. Sputum IL-17 and serum sFAS were measured using ELISA technique and eosinophils apoptotic ratio was detected by identification the morphological features of apoptosis after staining by Giemsa stain. The levels of sputum IL-17 and serum sFAS were increased in bronchial asthma patients than control group with statistically significant difference, there is decrease in eosinophils apoptotic ratio in bronchial asthma patients than healthy controls. Bronchial asthma patients have higher levels of sputum IL-17, serum sFAS and decreased eosinophil apoptotic ratio. Higher levels of IL-17 were associated with severity of the disease, high levels of serum sFAS inhibit the process of apoptosis and are associated with decrease in eosinophils apoptotic ratio.

Key words: Bronchial asthma, IL-17, serum sFAS, eosinophil apoptosis

INTRODUCTION

Asthma is a T cell driven chronic inflammatory disorder of the airways. Both T helper (Th2) and (Th1) lymphocytes, play an important role in the pathophysiology of asthma. Local overproduction of Th2 cytokines (IL-4, IL-5, IL-9 and IL-13) by Th2 cells in the asthmatic airways is well defined and recent studies indicate that Th1 cells, secreting IFN-γ, might cause severe airway inflammation.

IL-17 family members belong to a distinct category of cytokines that coordinate local tissue inflammation by inducing the release of pro-inflammatory and neutrophil-mobilizing cytokines. The importance of the IL-17 family in inflammatory and autoimmune disease is becoming increasingly apparent.

Recently, a separate T cell lineage, called Th17 cells or inflammatory T cells, producing IL-17A (or IL-17), has been identified as cellular source for IL-17.

Th17 cells might potentially play an important role in the pathophysiology of asthma. IL-17 is especially important for the recruitment of neutrophils and is expressed in bronchial biopsies, bronchoalveolar lavage fluid and sputum of patients with asthma. A role for IL-17 in murine asthma models has also been described and overexpression of IL-17 in lung epithelium causes chemokine production and leukocyte infiltration in vivo.

IL-17 expression in airways is upregulated upon allergen inhalation, and constitutes the link between allergen-induced T cell activation and neutrophil influx. Because neutrophils may be important in airway remodeling in chronic
severe asthma, targeting IL-17 may hold therapeutic potential in human asthma (5).

The plasma concentration of sFAS in asthmatic patients was significantly higher than that of the healthy control subjects. Since sFAS was shown to competitively inhibit the binding of membrane FAS antigen to FAS-ligand on the cell surface and, hence, to inhibit the Fas-mediated apoptotic pathway, the elevated plasma concentrations of sFAS in asthmatic patients might explain the down-regulated apoptosis of asthmatic T cells (6).

Administration of anti-FAS antibody induced apoptosis in the infiltrating eosinophils and abolished the increase in airway responsiveness to acetylcholine. Induction of apoptosis in eosinophils infiltrating asthmatic bronchi has a beneficial effect on airway hyperresponsiveness (7).

Serum sFAS levels of stable asthmatic patients were significantly higher than both the ones with acute attack and the controls. sFAS levels were higher in nonatopic asthmatics, both in the acute and stable phases, than those of the atopics. sFAS L levels during the acute attack were significantly higher than those of the controls. sFASL levels of patients receiving inhaler corticosteroid (ICS) treatment were significantly higher than those of the patients who did not receive ICS treatment during the acute attack. The significant increase of sFAS levels in asthmatic children during the stable phase of the disease rather than the acute phase made consideration of antiapoptotic mechanisms such as sFAS may have a great role in ongoing persistent chronic inflammation. The increased levels of sFASL in patients with acute asthma may be the reflection of the effort of immune system for limitation of inflammation by increasing apoptosis of inflammatory cells (8).

Many studies have shown an important role for apoptosis in the resolution and control of allergic inflammation. Induction of apoptosis is beneficial in the allergic response as it clears the airways of inflammatory cells like eosinophils and lymphocytes. Fas receptor-mediated eosinophil apoptosis is currently forwarded as mechanism resolving asthma-like inflammation (9).

Mild asthmatic subjects had a significantly lower percentage sputum eosinophils and a significantly higher eosinophil apoptotic ratio (AR) than moderate or chronic severe asthmatics. Severe asthmatic subjects had a significantly greater age, duration of asthma and sputum eosinophil count than mild asthmatic subjects. Asthmatic subjects' symptom scores, severity scores and age inversely correlated with AR and the percentage of sputum eosinophils. Baseline forced expiratory volume in one second inversely correlated with percentage sputum eosinophils and positively correlated with AR (10).

PATIENTS & METHODS

This study was carried out at Medical Microbiology & Immunology and Thoracic Medicine Departments, Mansoura University Hospitals from August 2007 to August 2009. Fifty patients with clinical diagnosis of bronchial asthma (history of any of the following: cough, worse particularly at night, recurrent wheeze, recurrent difficulty in breathing, recurrent chest tightness) and twenty healthy non smoker control subjects were enrolled in this study after informed consent. None of our subjects had any diseases such as DM, pneumonia, heart failure, respiratory tract infection during a month preceding the study.

All cases were subjected to the following:
- Full detailed medical history, including smoking history.
- Intake of drugs especially corticosteroid therapy.
- Full clinical examination.
- Chest X–ray postero-anterior and lateral views.
- Pulmonary function tests (spirometry, FEV1, FVC, FEV1/FVC) using computerized spirometry apparatus (JAEGGER Germany).
- Skin prick test using a group of allergens obtained from Ain Shams University Immunity Department to divide asthma patients into atopic non atopic.

-Sputum induction: sputum was induced with an aerosol of inhaled hypertonic saline using a modification of the method of Pin et al. (11). After pretreatment with inhaled salbutamol 200µg. The modification consisted of inhaling the hypertonic saline in concentrations of 3, 4 and 5% each for 7 min. Subjects were instructed to rinse the mouth with water and blow the nose after each inhalation to avoid contamination with saliva and postnasal drip. The sample was collected in a sterile container and was examined.

-Sputum processing for cytokine assay: Sputum specimens were collected in sterile containers and diluted with an equal amount of
isotonic saline and incubated for 15 min. at room temperature to digest mucus. Sputum digests were centrifuged at 1000 rpm for 3 min. to sediment cellular constituents. Cell supernatant were collected and stored frozen at -30°C until assessment of cytokine level.

**Measurement of IL-17 by ELISA in patients and control group using the RayBio® Human IL-17 ELISA kit.**

One hundred µl of standards, samples were added to the appropriate microtiter wells. After incubation for 2.5 hours, wells were aspirated and washed. One hundred 1 Biotinylated antibody were added to each well and incubated for 1 hour and thereafter, wells were thoroughly aspirated and washed. One hundred µl of TMB one step substrate reagent were added to each well. After incubation for 30 minutes, 50 µl of stop solution were added to each well. Absorbance of each well was read at 450 nm (using ELISA reader Spectra III, Austria) having blanked the plate reader against a chromogen blank.

**Measurement of serum sFAS in patients and control group using ELISA using the RayBio® Human FAS ELISA kit.**

One hundred µl of standards, samples were added to the appropriate microtiter wells. After incubation for 2.5 hours, wells were aspirated and washed. One hundred 1 Biotinylated antibody were added to each well and incubated for 1 hour and thereafter, wells were thoroughly aspirated and washed. One hundred µl of TMB one step substrate reagent were added to each well. After incubation for 30 minutes, 50 µl of stop solution were added to each well. Absorbance of each well was read at 450 nm (using ELISA reader Spectra III, Austria) having blanked the plate reader against a chromogen blank.

**Examination of sputum for apoptotic eosinophils**

Sputum samples were examined as soon as possible, within two hours. All sputum macroscopically free of salivary contamination was selected. the sputum was then mixed with equal volume of isotonic phosphate-buffered saline and incubation for a five minutes at room temperature was done. Sputum cells were separated by centrifugation at 800 rpm for 10 minutes. Slides were stained with Giemsa stain and 100 non-squamous cells were counted. the percentages were then averaged to give the final eosinophil counts. In addition, 100 eosinophils were counted under oil immersion lens and their morphology characterized as normal or apoptotic. Apoptotic eosinophils were differentiated on the basis of nuclear condensation, nuclear coalescence (shift from bilobed to mono-lobed nucleus) and cytoplasmic shrinkage. The differential white cell count was obtained and the eosinophil apoptotic rate was calculated as follows; Apoptotic Eosinophils (% of total white cells)/ Total eosinophil (% normal bilobed eosinophils) (12).

**Statistical Analysis**

The statistical analysis of data was done by using excel program and SPSS program statistical package for social science version 10. The description of the data done in form of mean (+/-) SD for quantitative data and frequency & proportion for qualitative data. For non parametric data as IL17 represented as median (minimum-maximum). The analysis of the data was done to test statistical significant difference between groups. For quantitative date, student t test was used to compare between 2 groups& One Way ANOVA and Bonferroni Post Hoc test to compare more than two groups. For non parametric data # Mann-Whitney Test was used to compare between 2 groups & Kruskal-Wallis Test is used to compare more than two groups. For qualitative data chi-square test was used. To test association between variables correlation co-efficiency test was used. N.B: P is significant if < or = 0.05 at confidence interval 95%.

**RESULTS**

The results of the present study showed higher levels of sputum IL 17 and serum sFAS in bronchial asthma patients in comparison to control group with statistically significant difference between both groups and positive correlation between sputum IL 17 and disease severity in addition to decrease in eosinophil apoptotic ratio in bronchial asthma patients.
Table (1): levels of sputum IL17, serum sFAS, sputum eosinophil percentage and sputum eosinophil AR in patients and control groups

<table>
<thead>
<tr>
<th></th>
<th>Patients (n=50)</th>
<th>Control (n=20)</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>mean</td>
<td>SD</td>
<td>mean</td>
</tr>
<tr>
<td>sFAS (pg/ml)</td>
<td>1192.34</td>
<td>795</td>
<td>367.64</td>
</tr>
<tr>
<td>Sputum eosinophils %</td>
<td>7</td>
<td>3.9</td>
<td>0.15</td>
</tr>
<tr>
<td>AR</td>
<td>0.61</td>
<td>0.2</td>
<td>.000</td>
</tr>
<tr>
<td>Sputum IL17 (pg/ml) Median(Min-Max)</td>
<td>65.8(24-487)</td>
<td>0(0-7)</td>
<td>&lt;0.001 #</td>
</tr>
</tbody>
</table>

# Mann-Whitney Test is used

Table (1) shows that Serum sFAS is higher in patients group compared to control group (P< 0.001). The table shows also that the percentage of eosinophils is higher in patients group compared to control group (P< 0.001) and AR is higher in patients group compared to control group (P< 0.001). levels of sputum IL17 in patients are higher compared to control group with statistically significant difference (P<0.001).

Table (2): levels of sputum IL17 in various degrees of disease severity

<table>
<thead>
<tr>
<th>Severity</th>
<th>Median (Min-Max)</th>
<th>P #</th>
<th>Mann-Whitney Test</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Severe</td>
</tr>
<tr>
<td>Mild</td>
<td>52.3(24.8- 90.2)</td>
<td>&lt;0.001***</td>
<td>&lt;0.001***</td>
</tr>
<tr>
<td>Moderate</td>
<td>62.4(45.5- 88.3)</td>
<td>&lt;0.001***</td>
<td>&lt;0.001***</td>
</tr>
<tr>
<td>Severe</td>
<td>405(354.7- 487.6)</td>
<td>&lt;0.001***</td>
<td></td>
</tr>
</tbody>
</table>

The previous table shows that levels of sputum IL 17 in severe asthma are higher than mild and moderate groups with statistically significant difference while the difference between mild and moderate groups is not statistically significant (p=0.365).

# Kruskal-Wallis Test is used

Table (3): levels of serum sFAS in various degrees of disease severity

<table>
<thead>
<tr>
<th>Severity</th>
<th>mean</th>
<th>SD</th>
<th>ANOVA</th>
<th>Bonferroni Post Hoc test</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Severe</td>
</tr>
<tr>
<td>Mild</td>
<td>639.5263</td>
<td>135.7352</td>
<td>&lt;0.001***</td>
<td>&lt;0.001***</td>
</tr>
<tr>
<td>Moderate</td>
<td>1017.3211</td>
<td>152.0051</td>
<td>&lt;0.001***</td>
<td>&lt;0.001***</td>
</tr>
<tr>
<td>Severe</td>
<td>2344.7500</td>
<td>850.5501</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Table (3) shows that levels of serum sFAS in severe asthma are higher than mild and moderate asthma and the levels are higher in moderate than mild group with statistically significant difference between the three groups with p value <0.001, <0.001 and =0.009 respectively.
Table (4): levels of sputum eosinophils AR in various degrees of disease severity

<table>
<thead>
<tr>
<th>Severity</th>
<th>Mean</th>
<th>SD</th>
<th>ANOVA</th>
<th>Bonferroni Post Hoc test</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mild</td>
<td>.8300</td>
<td>.06</td>
<td></td>
<td>&lt;0.001***</td>
</tr>
<tr>
<td>Moderate</td>
<td>.5795</td>
<td>.09</td>
<td>P &lt; 0.001***</td>
<td>&lt;0.001***</td>
</tr>
<tr>
<td>Severe</td>
<td>.3500</td>
<td>.11</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Table (4) shows that eosinophil AR is lower in severe group than moderate and mild groups with statistically significant difference between severe and mild (p<0.001), severe and moderate (p<0.001) and mild and moderate (p<0.001).

**DISCUSSION**

Bronchial asthma affects approximately 8% of the adult population and as many as 20% of children worldwide. Most of the studies have been conducted on patients with mild to moderate asthma and have shown airway inflammation to be associated with T-cell activation, eosinophil accumulation, and Th2-type cytokine production. Severe asthma represents 10% of the asthmatic population but has greater morbidity and mortality and uses a disproportionate amount of health care expenditures. Furthermore, the subpopulation of patients with severe asthma appears to manifest a different pattern of airway inflammation that is not associated with either classic Th1 or Th2 cells. Th17 cells produce a number of cytokines, but in particular IL-17A and IL-17F. These cells have been implicated in the pathogenesis of a number of diseases, including Bronchial asthma (13).

In the present study, we have investigated the levels of IL 17 in induced sputum in bronchial asthma patients and healthy controls. We found higher levels of this cytokine in bronchial asthma patients median=65.8(24-487) in comparison to healthy control median=0 (0-7) (p<0.001), with higher levels in severe asthma group median = 405(357.7-487.6) than mild 52.3 (24.8-90.2) and moderate 62.4(45.5-88.3) asthma groups(p<0.001). All healthy individuals had low to undetectable IL 17 in their sputa which makes increased IL17 levels potentially useful as diagnostic aid in asthma, also there is positive correlation between the levels of IL 17 and the eosinophil percentage in the induced sputum from asthmatic patients (r=0.904, p<0.001) which may suggest eosinophils as cellular source of IL 17.

Our results concur that of Shen et al. (14) who demonstrated that the concentrations of IL-17 both in patients with COPD during acute exacerbation and with asthma were significantly higher than that in the control subjects (P<0.001). The levels of IL-17 in patients with COPD during acute exacerbation positively correlated with that of IL-8 (r =0.381, P=0.038) and with the percentage of neutrophils (r = 0.446, P = 0.010) respectively. There was also a positive correlation between the concentrations of IL-17 and the numbers of eosinophils in patients with asthma. The concentrations of IL-17 in patients with acute exacerbation of COPD and in patients with asthma were significantly increased. IL-17 may play a role in the airway inflammation in both COPD and asthma.

Molet et al. (15) demonstrated that IL-17 is upregulated in asthma and eosinophils also are cellular sources of its production, and that IL-17 increases synthesis of IL-6 and IL-11 by bronchial fibroblasts derived from bronchial biopsies of asthmatic subjects. Because IL-17 is produced mainly by CD4+ T cells and stimulates the release of fibroblast-derived mediators, it is hypothesized that IL-17 has a potential role in asthma, in which T cells and stromal cells are thought to play a key role in the progression of the disease. IL-17 might be a potential player in the cytokine network involved in asthma, and there is possible indirect effect of IL-17 on airway remodeling. Cells positive for IL-17 immunoreactivity were found in sputum and BAL fluid of both asthmatic and normal control subjects. The numbers of cells expressing IL-17 immunoreactive protein were significantly higher in sputum (P<0.001) and BAL (P <0.005) recovered from asthmatic subjects than in sputum and BAL recovered from nonasthmatic controls. The morphology of most
of the IL-17-immunoreactive cells was consistent with eosinophils.

In the present study, we measured the plasma concentration of sFAS in asthmatic patients and control group using (ELISA) technique. The results showed higher levels of sFAS in bronchial asthma patients (1192.34 ± 795) than control group (367.64 ± 41.6) with statistically significant difference between the two groups (P<0.001), higher levels of sFAS was detected in severe asthma group (mean = 2344.750 ± 850.5501) than mild (639.5263 ± 135.7352) and moderate groups (1017.3211 ± 152.0051) with statistically significant difference between severe and mild (p< 0.001) and severe and moderate (p< 0.001) and mild with moderate groups (p=0.009).

The results are in agreement to that of Ho et al.(6) who demonsrted that the plasma concentration of sFAS in asthmatic patients was significantly higher than that of the healthy control subjects (median, 3. 1 ng/mL [IQR, 2. 5 to 3. 9 ng/mL] vs 2. 6 ng/mL [IQR, 2. 2 to 2. 9 ng/mL], respectively; p = 0. 033).

Since sFAS was previously shown to competitively inhibit the binding of membrane FAS antigen to FAS-ligand and on the cell surface and, hence, to inhibit the FAS-mediated apoptotic pathway, the elevated plasma concentrations of sFAS in asthmatic patients might explain the down-regulated apoptosis of asthmatic T cells. Related study of İkinciogullari, et al.(8) measured serum sFAS and sFASL levels by (ELISA), during and 2 weeks after the treatment of the acute attack in 15 asthmatic children (range, 2–14 years; median, 5 years) to evaulate the possible role of the FAS-FASL system in asthmatic inflammation. Serum sFAS levels of stable asthmatic patients were significantly higher than both the ones with acute attack and the controls (p<0.001, p<0.01, respectively). sFAS levels were higher in nonatopic asthmatics, both in the acute and stable phases, than those of the atopics. sFASL levels during the acute attack were significantly higher than those of the controls (p<0.05) sFASL levels of patients receiving inhaled corticosteroid treatment were significantly higher than those of the patients who did not receive inhaled corticosteroid treatment during the acute attack (p<0.05). The significant increase of sFAS levels in asthmatic children during the stable phase of the disease rather than the acute phase made us consider that antiapoptotic mechanisms such as sFAS may have a great role in ongoing persistent chronic inflammation. The increased levels of sFASL in patients with acute asthma may be the reflection of the effort of immune system for limitation of inflammation by increasing apoptosis of inflammatory cells.

In the present study we investigated the eosinophil apoptosis by detection of eosinophil apoptotic ratio (AR) in induced sputum from patients with bronchial asthma and control group. We found that eosinophil AR in bronchial asthma patients is 0.61± 0.2 while in control control group the mean of this ratio is 0.80 ± 0.06 and moderate groups (mean=0.5795 ± 0.09) with statistically significant difference between these groups (P<0.001). Also we found positive correlation between eosinophil AR and FEV1(r=0.815, p = <0.001).

This result is in agreement with that of the study of Duncan et al,(10) who demonstrated a relationship between reduced sputum eosinophil apoptosis and increased clinical severity of chronic stable asthma, providing additional evidence that eosinophil apoptosis may be important in the resolution of eosinophilic airway inflammation in asthma, the study showed that mild asthmatic subjects had a significantly lower percentage sputum eosinophils (mean7.4) and a significantly higher eosinophil apoptotic ratio (0.8) than moderate (34.5% and 62 respectively) or chronic severe asthmatics(50.5% and0.49 respectively). Severe asthmatic subjects had a significantly greater age, duration of asthma and sputum eosinophil count than mild asthmatic subjects (P<0.05) Asthmatic subjects' symptom scores, severity scores and age inversely correlated with AR and the percentage of sputum eosinophils. FEV1 inversely correlated with percentage sputum eosinophils and positively correlated with AR.

In conclusion, this study has demonstrated a relationship between sputum IL-17, eosinophil apoptosis, serum FAS and clinical severity of asthma which direct the attention of therapeutic trials to these parameters.

REFERENCES


الملخص العربي


يعتبر مرض الزوو الشعبي من الأمراض انتشاراً واسعاً على مستوى العالم إذ تبلغ نسبة الإصابة به حوالي 8% في الكبار و 20% في الأطفال، وتختلف شدة المرض في الحالات المختلفة حيث تصل حالات الشدمة 10% من حالات المرض، وتشكل نسبة عالية من الوفيات. كان الهدف من هذه الدراسة إعداد مستويات إرتالوكين في عينات بصل مرضى الزوو الشعبي وكذلك مستويات الفاسكو. وُجِد من خلال هذه الدراسة أن تأثير المرض على النموات الخلايا ذات الصبغة الحمراء باعتبارها من أهم الخلايا المثيرة في عملية заболевания المرضية. حيث أن هذه الدراسة التي اكتملت في أغسطس 2009 وشملت 50 حالة من حالات الزوو الشعبي بدرجات المرض المختلفة المتوسطة والشديدة و20 حالة من الأصحاء غير المحتملين كمجموعة المقارنة تم إعداد مستويات إرتالوكين 17 والفاس باستخدام طريقة الإلزام و كذلك حساب نسبة المرضى لخلايا ذات الصبغة الحمراء إعتماداً على الحصص الشكلية للخلايا الحمضية للموت المبرمج بعد صعوبة مريحة. كما قام وضع قاعدة المرضى السرية للمساعدة في تفسير شدة المرض وكذلك اختبار الجسدية للكشف عن المرض، ويتم استخدام هذه المعلومات في حالات الزوو الشعبي ذات الجسدية، والتي تؤدي إلى تطبيق علاجات فعالة للمرض، وتأتي الأثر المعنوي على حالة المريض في يؤدي إلى تحسين جودة حياة المريض. وقد أظهرت هذه الدراسة ارتفاع مستويات إرتالوكين والفاسكو في حالات الزوو الشعبي مما يشير إلى تأثر هذه الحالات بالاختلالات الإخراجية التي تؤدي إلى تأثيرات على النموات الخلايا ذات الصبغة الحمراء في مرضى الزوو الشعبي. حيث كانت نسبة الأمراض الشديدة بين حالات الزوو الشعبي ونسبة الأمراض المشابهة في حالات الزوو الشعبي. هذا نتيجة لعملية الممرض ووسائل العلاجية. في مرضى الزوو الشعبي.