Increase of Bcl-2/Bax ratio correlated with decrease of lymphocyte apoptosis: A study in the bronchiolus and lung of asthmatic mice

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The failure of lymphocyte apoptosis is one of the factors causing chronic airway inflammation in asthma. Some studies indicate the role of Bcl-2 in inhibition of lymphocyte apoptosis, but still little research is on the role of Bax and its relationship to Bcl-2 in asthma. The purpose of this study was to prove the role of Bcl-2-lymphocyte in inhibition of lymphocyte apoptosis and decrease in Bax-lymphocytes in bronchiolus and lung of asthmatic mice. This study was a randomized control group design. Subjects were Balb/c mice which divided into 2 groups: non-asthma and asthma. Asthma group were sensitized with ovalbumin intraperitoneally on day 0 and 14, followed by inhalation every 2 to 3 days for 6 weeks. At week 8, all subjects terminated. Bcl-2 and Bax-lymphocytes expression were examined with immunohistochemical method, whereas apoptotic lymphocytes by Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) method. Statistical analysis used was the independent sample t-test and regression analysis, with 95% confidence interval. Bronchial and lung specimens were obtained from 18 subjects (9 from each group). The ratio of apoptotic lymphocytes decreased in the asthma group (p = 0.003), Bcl-2-lymphocytes increased in the asthma group (p < 0.001), and Bax-lymphocytes decreased in the asthma group (p = 0.003). There was a strong negative correlation (r = -0.66, r² = 0.43, p = 0.003) between the Bcl-2 and lymphocyte apoptosis. There was also a strong negative correlation (r = -0.56, r² = 0.35, p = 0.009) between the ratio Bcl-2/Bax and lymphocyte apoptosis. However, no significant relationship was found between Bax-lymphocytes and lymphocyte apoptosis (r = 0.36, r² = 0.13, p = 0.15). Increasing the ratio of Bcl-2/Bax inhibit lymphocyte apoptosis where Bcl-2 plays more role than Bax.

Key words: Asthma, ovalbumin, lymphocyte apoptosis, Bcl-2, Bax.

INTRODUCTION

Asthma is a chronic inflammatory disorder of respiratory tract with many cells that play a role, especially mast cells, basophils, and lymphocytes T. Asthma is a problem throughout the world with a prevalence estimated at 300 million people (1 to 18% of the population in various countries and 10% in children), with mortality reaching 250,000 people per year. Chronic inflammation is associated with respiratory tract hyperresponsiveness that causes recurrent episodes of wheezing, shortness of breath, feeling heavy in the chest (chest tightness), and...
cough particularly at night or early morning (GINA Executive Committee, 2009; UKK Pulmonologi, 2004).

Failure to ease the inflammatory process after the occurrence of exacerbations is a major problem in asthma. Chronic or persistent inflammation has been proved as one of the main factors that influence the severity and frequency of exacerbations, and has a role in airway remodeling, smooth muscle hypertrophy, and airway hyperreactivity (Busse and Lemanske, 2001; Tong et al., 2006). One of the mechanisms that suspected playing a role in the chronicity of asthma is the failure of inflammatory cells apoptosis, such as: eosinophil, neutrophil, T lymphocytes, and macrophages (Lamb et al., 2005; Muller et al., 2006; Spinozzi et al., 2008). Physiologically, apoptosis is a programmed cell death which is a form of cell death to maintain the balance of the development of body cells. Apoptosis occurs only in some cells in small quantities, and selectively (Guicciardi and Gores, 2005).

In the inflammation process, apoptosis serves to control the "excess" of inflammatory cells, limiting tissue damage, and ease the inflammation process (Spinozzi et al., 2008). Research on several diseases, for example nasal polyps and rheumatoid arthritis, suggest that the chronicity of the disease is associated with failure or delay apoptosis in inflammatory cells and that these cells survive in inflammation places (Salmon et al., 1997; Simon et al., 1997). T lymphocytes, especially Th2 cells, have a central role in the regulation of the immune system.

One of the factors that play a role in apoptosis of T lymphocytes is pro-and anti-apoptotic proteins. Bax is a pro-apoptotic proteins that act as an executor protein in mitochondrial channels of apoptosis (intrinsic pathway) so that its expression can be used as an apoptotic marker, whereas expression of Bcl-2 showed the existence of apoptosis inhibitors associated with the remodelling process (Akbar et al., 1996; Kim et al., 2006). This event was reinforced by the evidence of increased Bcl-2 expressions which was proportional to the number of T lymphocytes that infiltrate into the bronchial submucous, and also comparable with the severity of asthma. In addition, Bcl-2 mRNA in asthma were found in T lymphocytes (Hamzaoui et al., 1999; Vignola et al., 1999). There was another evidence which states that Bcl-2 can inactivate Bax (Kim et al., 2006). Therefore, we perform an in vivo study to examine the influence of pro-and anti-apoptosis proteins in lymphocyte apoptosis.

MATERIALS AND METHODS

Experimental animals

Experimental animals were Mus musculus mice (Balb/c) obtained from Veterinaria Farma experimental animal cages center at Ahmad Yani Street Surabaya. The sex of mice selected was female because it has a better response to allergens than male mice (Epstein, 2004). Mice inclusion criteria were: age 6 to 12 weeks, weight 20 to 30 g, and healthy (good appetite and activity, fur did not fall out). Experimental animals were excluded if diagnosed to be ill during observations, which appear from the decrease in activity and other important clinical signs (weight loss, breath pattern, diarrhea and vomiting), or died.

Study design

This research was a true experimental study design with randomized control group, performed at the Laboratory of Pharmacology and Biomedics, Faculty of Medicine, Brawijaya University for 7 months, in February, 2009 to September, 2009. The large size of the sample was determined by: \( p \) = number of subjects per group, \( n \) = number of research group (Hanafiah and Ali, 1991). Based on this formula, by using 2 groups of studies, research samples required were 8 samples per group. Samples that meet the inclusion criteria will be divided into 2 groups: treatment group (asthma) and control groups (non-asthma) with simple random sampling technique.

Initial sensitization was injected intraperitoneally by injecting a mixture of 10 μg ovalbumin (OVA) + 1 mg Al(OH)₃ dissolved in 0.5 ml of normal saline (NaCl 0.9%) on day 0 and day 14.

Inhalation sensitization tests was conducted by giving ovalbumin (OVA) 1% in 8 ml normal saline (NaCl 0.9%) per treatment using OMRON Nebulizer type NU-017 for 20 min, with air flow volume and nebulization volume on a scale of 1. Sensitization by inhalation was repeated for 6 weeks. After 6 weeks, all experimental animals in both groups were terminated. The pulmonary organs placed in the organs storage device and fixed in formalin 10%, and then made for histopathology preparations.

Ovalbumin (OVA)

OVA used in this study were ovalbumin with SERVA brand. This OVA was an albumin egg lyophil salt-free and lysozyme-free produced by SERVA electrophoresis GmbH. Aluminium hydroxide (alum) was used as an adjuvant (Zosky et al., 2007).

Microscopic observation

Each tissue sample was made for slide preparation. There were 18 slides, consisting of 9 slides from each group. Slide examination was conducted covertly (blinding) by 2 separate biomedical analyst. Examination and calculation of apoptotic lymphocytes, Bcl-2 expression, and Bax expression were performed on each slide using 1000× magnification, and calculated per mm² and the average value taken.

Lymphocyte apoptosis observation

Staining technique of lymphocyte apoptosis was done using TUNEL labeling of fragmented DNA (Apo-BrdU-IWC CatK403-50:Lot P10013). The number of apoptotic lymphocytes is the number of lymphocytes per mm² (single core cell, nucleus and cytoplasm ratio 3:4, with a red cytoplasm), with dark brown cell nuclei after staining with TUNEL (Lamb et al., 2005).

Bcl-2 dan Bax-limfosit expression

Measurement of Bcl-2 and Bax expression was done using rabbit polyclonal anti-Bcl-2 (Cat. # MS-598 - P0 LabVision) and anti-Bax
Table 1. Experimental animal characteristic.

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Treatment group</th>
<th>Control group</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Type</td>
<td><em>Mus musculus</em> (balb/c mice)</td>
<td><em>Mus musculus</em> (balb/c mice)</td>
<td></td>
</tr>
<tr>
<td>Number</td>
<td>9</td>
<td>9</td>
<td></td>
</tr>
<tr>
<td>Age (week)</td>
<td>6-12</td>
<td>6-12</td>
<td>p = 0.751</td>
</tr>
<tr>
<td>Sex</td>
<td>Female</td>
<td>Female</td>
<td></td>
</tr>
<tr>
<td>Weight (g)</td>
<td>26.82±2.19</td>
<td>26.36±3.36</td>
<td></td>
</tr>
</tbody>
</table>

On the characteristics of experimental animals, there were no differences in animal species, number, age, gender, and body weight.

IgE OVA examination

Measurement of IgE OVA was by ELISA method with anti IgE biotin conjugate (Santa Cruz cat# sc-66169). The result was read using ELISA reader at a 450 nm wavelength.

Statistical analysis

The average (mean) ratio of lymphocyte apoptosis, expression of Bcl-2 and Bax lymphocytes between two groups (treatment and control) were analyzed using independent-sample T-test. The relationship between expression of Bcl-2 lymphocytes and ratio of lymphocyte apoptosis, Bax expression ratio of lymphocytes and lymphocyte apoptosis and Bcl-2/Bax expression ratio and ratio of apoptotic lymphocytes were analyzed using correlation and linear regression test, with the value of confidence interval at 95%. All data were processed using statistical package for social sciences (SPSS) 17.0 for windows.

Research approval

Research was conducted after getting approval by the Commission on Health Research Ethics of Saiful Anwar Hospital Malang.

RESULTS

Samples characteristic

This study used 6 to 12 week-old female Balb/c mice. Based on the calculation, the number of samples needed was ≥ 8.5 samples per group, so we used 9 mice in each group (total of 18 mice). The mean of mice body weight was 26.82 g in the treatment group (asthma), and 26.36 g in the control group (non-asthma) (Table 1).

Comparison of Bcl-2 lymphocyte expression, Bax lymphocyte ratio, lymphocyte apoptosis and IgE OVA between experimental and control group

From the result, it was found that the expression of Bcl-2 was higher in the treatment group (asthma) than the control group (Figure 2). Conversely, the Bax expression was significantly lower in the treatment group than the control group (Figure 3). In observation of apoptotic lymphocytes, it was found that the ratio of apoptotic lymphocytes was lower in the treatment group than the control group (Figure 1). On the measurement of OVA and total IgE levels, a high level was found in the treatment group. There was a negative relationship between expression of Bcl-2 and the ratio of apoptotic lymphocytes, where the ratio of lymphocyte apoptosis is influenced by Bcl-2 expression by 43%. The ratio of apoptotic lymphocytes could be predicted by the formula: The ratio of apoptotic lymphocytes = 0.96 to 0.06 (expression of Bcl-2) (Figure 3). On the other hand, there was no significant correlation between Bax expression ratio of lymphocytes and lymphocyte apoptosis. There was a negative relationship between the ratio of Bcl-2/Bax expression and lymphocyte apoptotic ratio, where the ratio of lymphocyte apoptosis is influenced by the ratio of Bcl-2/Bax expression by 31%. The ratio of apoptotic lymphocytes could be predicted by the formula: The ratio of apoptotic lymphocytes = 0.66 to 0.21 (the ratio of Bcl-2/Bax expression) (Figure 3).

DISCUSSION

Provision of ovalbumin (OVA) in experimental animals (rats) will trigger a systemic allergic response with elevated levels of total IgE and specific IgE OVA (Tumes et al., 2008). In this study, a significant difference was found between the two groups: OVA IgE levels were higher in the asthma group (p = 0.001) (Table 2). Increased expression of IgE OVA proved that true asthma occured in the treatment group. Sensitizing antigen on normal airway immune response will cause proliferation or clonal expansion of lymphocytes and will be followed by apoptosis of lymphocytes immediately after peak phase of clonal expansion, a mechanism called activation induced cell death (AICD) (Krammer, 2000; Spinozzi et al., 2008). In asthma, there was an apoptotic T cell dysfunction. Selective resistance to apoptosis of CD4 + T cells in patients with asthma led to the occurrence of T-cell-dependent immunoinflammation...
in asthma. It was suspected that asthma could be due to decreased elimination of activated T cells and increased recruitment and activation of T cells. Thus, the phenotype of T cells was found consistently in asthma patients (Pierce et al., 2007).

In this study, there was a difference in the ratio of lymphocyte apoptosis in the group who experienced asthma (allergy) and non-asthma (p = 0.003) (Table 2). Cell death is necessary to maintain T lymphocyte homeostasis. Failure in cleaning the activated cells will prolong immune response and cause chronic inflammation (Abdulamir et al., 2009; Hildeman et al., 2002). Asthma is a chronic inflammation with characterization of activated T cell in peripheral blood vessels and airway. There was a direct correlation between numbers of activated T lymphocyte in bronchial mucous and severity of asthma. In allergen induction, T lymphocyte CD4+ was recruited selectively to bronchial mucous. This recruitment was accompanied by decrease number of T lymphocyte in circulation (Abdulamir et al., 2009; Darveau et al., 2008).

Apoptosis is regulated by intrinsic point of several proteins, including Bcl-2 and Bcl-XL. Apoptosis is triggered by activation of caspase-9 and caspase-3, and can be inhibited by the expression of Bcl-2 (Adams, 1998; Darveau et al., 2008; White and Dorscheid, 2002). In this study, proved levels of Bcl-2 lymphocytes was higher in asthma group (p < 0.001) (Table 2). In addition, beside the increased expression of anti-apoptotic protein, apoptosis failure in asthma is also caused by decreased expression of pro-apoptosis proteins such as Bcl-2-associated X protein (Bax) and Fas/FasL (Abdulamir et al., 2009; Darveau et al., 2008), whereas Bax is an executor of apoptosis proteins and can be used as a marker apoptosis (Kim et al., 2006). This study proved that lymphocytes Bax expression was lower in the asthma group (p = 0.003) (Table 2).

Although there was a decrease Bax apoptotic lymphocytes and lymphocytes in asthma group, however our study found no significant correlation between Bax expression lymphocytes with lymphocyte apoptosis (r = 0.36, $r^2 = 0.13$, p = 0.15) (Table 3). Possible cause of the existence of a weak association was due to experimental animals used in this study which are strains of Balb/c mice. Previous studies using this strain of mice also found increased antiapoptosis protein Bcl-2 and Bcl-XL without a decrease in antiapoptosis protein Bax after sensitization with ovalbumin, unlike the case of used mice strains CBA/Ca where apoptosis is obtained more quickly because the protein expression proapoptosis Bax (Tumes et al., 2008). Second possibility is the presence of other factors that play a role in lymphocyte apoptosis rather than Bax lymphocytes. This suggests that inhibition of apoptosis of lymphocytes in asthma not only played by Bax, but probably by a complex interaction of many factors.

Increased expression of protein anti-apoptosis proteins, especially Bcl-2 as it has been proven in this study, is one

**Table 2.** Level of IgE OVA, lymphocyte apoptotic ratio, Bcl-2 and Bax expression in treatment and control group.

<table>
<thead>
<tr>
<th>Variable</th>
<th>Treatment group (Mean ± SD)</th>
<th>Control group (Mean ± SD)</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bcl-2</td>
<td>13.06±1.67</td>
<td>7.22±1.72</td>
<td>p&lt;0.001</td>
</tr>
<tr>
<td>Bax</td>
<td>6.11±1.08</td>
<td>8.94±2.20</td>
<td>p =0.003</td>
</tr>
<tr>
<td>Apoptosis ratio</td>
<td>0.14±0.08</td>
<td>0.54±0.34</td>
<td>p =0.003</td>
</tr>
<tr>
<td>IgE OVA</td>
<td>11.22±2.30</td>
<td>7.60±1.13</td>
<td>p =0.001</td>
</tr>
</tbody>
</table>

**Table 3.** Corelation between Bcl-2, Bax, Bcl-2/Bax expression ratio and lymphocyte apoptotic ratio.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Lymphocytes apoptosis ratio (cell/mm²)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>r=0.66</td>
</tr>
<tr>
<td>Bcl-2 expression (cell/mm²)</td>
<td>$r^2=0.43$</td>
</tr>
<tr>
<td></td>
<td>$y=0.96-0.06x$</td>
</tr>
<tr>
<td>Bax expression (cell/mm²)</td>
<td>r=0.36</td>
</tr>
<tr>
<td></td>
<td>$r^2=0.13$</td>
</tr>
<tr>
<td>Bcl2/Bax expression ratio</td>
<td>r=0.56</td>
</tr>
<tr>
<td></td>
<td>$r^2=0.31$</td>
</tr>
<tr>
<td></td>
<td>$y=0.66-0.21x$</td>
</tr>
</tbody>
</table>
of the factors that cause barriers to lymphocyte apoptosis. Other factors were not examined in this study, including expression of anti-apoptotic protein Bcl-XL, expression of Fas/Fas-L, Bak, and the proteins pro-apoptotic subclass III (BH3-only proteins).

Bcl-XL is a Bcl-2 homologue. Experiments in vivo in rats showed that during the onset of inflammation, there was increased expression of Bcl-XL along with significant decrease in Bax expression. In the same experiment, inhibition of Bax by V5 (Bax inhibitory peptide) results in prolonged inflammation, supporting the hypothesis that Bax has an important role in the induction of apoptosis of inflammatory cells, potentially accelerating the resolution of inflammation (Hallett et al., 2008).
The role of Fas/Fas-L in regulating lymphocyte apoptosis has been demonstrated by Tong et al. (2006) through studies in vivo in Fas deficient mice, in which the number of lymphocytes was higher than in normal mice up to day 14 after exposure to allergens, with the dominant cytokines TH2. It was also demonstrated in vitro that administration of IL-4 on T-lymphocyte cell cultures decreased the Fas expression. This suggests that the regulation of lymphocyte apoptosis was not only through the intrinsic (mitochondrial) pathway, but also the extrinsic (death receptor) pathway in which the deficiency of Fas/Fas-L is likely to be more involved in the inhibition of apoptosis.

Besides Bax, Bak also had a big role in the inhibition of mitochondrial apoptosis path as evidenced in research by Lindsten et al. (2000). Mice with a deficiency of either Bax or Bak only experienced mild abnormalities in development, whereas in mice with deficiency of both Bax and Bak, 90% experienced perinatal mortality, while 10% had multiple abnormalities. This suggests that these two proteins two proteins have complementary roles in tissue homeostasis. However, further research is still needed because there is no research that examines the role of Bak in apoptotic lymphocytes in asthma.

There is a clear balance between the pro- and anti- apoptotic proteins in the process of inflammation linkage (Hallett et al., 2008). Pro-and anti-apoptosis which influenced apoptosis of lymphocytes in this study were described by using the ratio of Bcl-2/Bax. In linear regression analysis, it was a significant negative relationship between the ratio of Bcl-2/Bax expression and lymphocyte apoptosis ratio, where the ratio of lymphocyte apoptosis is influenced by the ratio of Bcl-2 expression of lymphocytes by 31% \( (r = -0.56, r^2 = 0.31; p = 0.017) \) (Table 3). Apoptosis of lymphocytes can be predicted by the formula: Lymphocyte apoptosis ratio = 0.66 to 0.21 (the ratio of Bcl-2/Bax expression.) This indicates that an increase in Bcl-2/Bax ratio will decrease the apoptosis of lymphocytes. Because the increased Bcl-2/Bax ratio of 31% only had a role in reducing apoptosis of lymphocytes, mean decrease in apoptosis of lymphocytes in this study was also caused by pro-and anti-apoptosis factor proteins.

Studies conducted by Abdulamir et al. (2008) found that the Bcl-2/Bax ratio was higher in severe asthma compared with mild asthma and healthy controls. The number of leukocytes in peripheral blood cells also increased with increasing ratio of Bcl-2/Bax. Bcl-2/Bax ratio is associated with asthma severity and confirmed that the protein Bcl-2 can inhibit the expression and effects of pro-apoptotic protein Bax. Progressive decrease in apoptosis resulted in increased severity, chronicity, and persistence of inflammation in asthma.

This study showed that there was inhibition of apoptosis of lymphocytes in asthma, which is influenced by a variety of complex factors interaction. The existence of various factors that influence the apoptosis of lymphocytes in this study, due to the design of in vivo studies that illustrate the pathogenesis and patho-physiology of asthma, are more similar to the mechanism of the real disease. Partially, this research was also able to describe the pathogenesis of asthma from the point of failure of apoptosis. This research was able to demonstrate the role of intrinsic point (especially Bcl-2 lymphocytes) in the inhibition of apoptosis of lymphocytes in order to consider the use of asthma therapy via this route.

In this experiment, female Balb/c mice was chosen because they have better response to allergens (Epstein, 2004) and act as representative to provide a snapshot of asthma in humans. This animal model showed a Th2-mediated allergic inflammation, airway mucosal eosinophilia, and airway hyper-responsiveness (Nials and Uddin, 2008), and also showed a goblet cell hyperplasia, epithelial hypertrophy, and sub-epithelial fibrosis and peribronchial. These phenomena were settled after cessation of exposure to allergens, and airway remodeling picture were obtained (Kumar et al., 2004; McMillan and Lloyd, 2004). In this study, the asthma group can be analoged to the description of asthma in humans in the form of airway sensitization in mice and is characterized by an increase in IgE ovalbumin (Table 2).

There are limitations in our study: (1) parameter examination were conducted only once after chronic exposure to ovalbumin; (2) determination of lymphocyte was based solely on morphology (not using a marker, so the lymphocyte subsets of lymphocytes observed were not known); (3) there are limitations of immunohistochemistry in the determination of cells expressing Bcl-2 and Bax; and (4) other factors that influence lymphocyte apoptosis was not examined. Therefore, further research is necessary to: (1) perform serial examination starting from initial exposure until the exposure end (chronic exposure), (2) examine marker(s) lymphocytes to determine the subset of lymphocytes (mainly CD4 + T cells), (3) examine technique with flow cytometry as it can be a measured expression of Bcl-2 and Bax along with cells that are expressed, and (4) examine other factors that may affect lymphocyte apoptosis, among others: IFN-γ, Fas/Fas-L, Bak, pro-and anti-apoptosis proteins.

**Conclusion**

This study showed an increased expression of Bcl-2 lymphocytes, decreased Bax expression and decreased lymphocyte ratio of lymphocyte apoptosis in mice with asthma. Increased expression of Bcl-2 decreases the ratio of apoptotic lymphocytes by 43%. However, the
decreased expression of Bax was not significantly associated with decreased lymphocyte apoptosis. Increased expression of Bcl-2/Bax ratio decreases the ratio of apoptotic lymphocytes by 35%. This shows that increasing the ratio of Bcl-2/Bax inhibit lymphocyte apoptosis, where Bcl-2 play more roles than Bax.

REFERENCES


Refferences