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Bcl-2 Protein Expression in Pulmonary Specimens of Sulfur Mustard Victims

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ABSTRACT

***Background**: Considering the role of sulfur mustard gas in development of acute and chronic pulmonary complications and the role of some genes including Bcl-2 in pulmonary fibrosis, we decided to study Bcl-2 gene expression in lung biopsy specimens of victims in comparison with normal lung.*

***Materials and Methods**: Lung biopsy specimens were taken from 13 sulfur mustard (SM) victims with pulmonary complication and were stained by Hematoxylin and Eosin (H & E) and avidin-biotin methods. We used normal lung blocks from forensic medicine as the normal group. These blocks were treated and stained with the same procedure as the case group. Both groups' blocks were studied by a pathologist simultaneously.*

***Results**: The percentage of macrophages in sulfur mustard victims' specimens was significantly lower than that of the control group. There was also a significant increase in lymphocytes of victims in comparison with the normal group.*

Neutrophil percentage and Bcl-2 protein in these cells showed no significant difference between the two groups. Bcl-2 protein in fibroblasts and epithelial cells of sulfur mustard victims was significantly higher than the control group.

Conclusion**: According to the results of this study in comparison with the results of patients with idiopathic pulmonary fibrosis (IPF), pulmonary fibrosis can be confirmed as one of the late complications of SM victims. According to these results, up-regulation of Bcl-2 protein and subsequent alveolar epithelial cell hyperplasia and proliferation of fibroblasts may be a mechanism for pulmonary fibrosis induced by mustard gas. It could be important for developing new strategies for the treatment of fatal complications of this chemical warfare.* ***(Tanaffos 2008; 7(1): 25-31)

***Key words**: Sulfur mustard, Pulmonary fibrosis, Bcl-2, Apoptosis*

INTRODUCTION

Sulfur mustard (2,2'-dichloroethyl sulfide) is an alkylating agent which was used as a chemical warfare first in World War I (1). Its latest use was reported to be in Iraq-Iran war (2-4). Skin, eyes, upper and lower airways injuries are of the acute complications of exposure to this gas because these organs are directly exposed to the gas (5).

Despite acute complications, exposure to this gas can cause chronic and sometimes progressive

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complications the most important of which being pulmonary complications such as asthma, chronic bronchitis (6-10) and pulmonary fibrosis (11). Although several studies have been performed in this regard, the mechanism of this gas causing acute and chronic pulmonary complications is still unknown (12). However, some hypotheses have been suggested including DNA damage, lysosome instability and Ca^{+2}-related toxicity. It seems that DNA is the main target of this gas in causing complications (13). Pulmonary fibrosis is an important life-threatening complication of sulfur mustard which sometimes results in victims' death. Therefore, understanding the cellular and molecular pathways resulting in this complication is a matter of great importance so that the pharmacological intervention for its prevention and treatment would be feasible (14).

One of the suggested mechanisms in development of pulmonary fibrosis is induction of apoptosis in epithelial cells and decreased apoptosis in lung fibroblasts (15,16).

Apoptosis (programmed cell death) is an active form of cell death and is characterized by cell pyknosis, fragmentation of the chromatin and budding of the plasma membrane (17) and is regulated by different genes such as P53, bax and Bcl-2 (18). Among these, Bcl-2 is the first gene recognized in the process of apoptosis which is an inhibitor for this process and extends cell lifespan (19,20).

Bcl-2 gene has been evaluated as one of the genes responsible in pulmonary fibrosis during the recent years. Considering the important role of this gene in induction of apoptosis and considering the confirmed role of apoptosis in development of pulmonary fibrosis, study in this regard is still going on (21, 22).

Noting the high rate of chronic pulmonary complications in victims exposed to mustard gas and development of fibrosis, a main life-threatening pulmonary complication in these patients, we decided to evaluate Bcl-2 gene expression in patients' lung biopsy specimens to understand cellular and molecular pathways in development of pulmonary complications due to this gas and finding a way for pharmacological intervention for prevention and treatment of this complication.

MATERIALS AND METHODS

This was a case-control study and by considering a confidence interval=95%, 13 sulfur mustard victims suffering late pulmonary complications were selected by easy sampling. The inclusion criteria were no use of any oral or systemic corticosteroids during the last 3 months, absence of any infectious pulmonary disease and no cigarette smoking. After obtaining consent, the patients underwent fiberoptic bronchoscopy (Olympus BFIT, Japan) by a specialist. Upper airways were desensitized by using lidocaine 2% and then 0.5 mg atropine was injected IM and lung specimen was taken by transbronchial biopsy from the middle lobe of the right lung and were fixed. Paraffin embedded blocks were prepared and 2-3 micron tissue sections were prepared by microtome.

Samples were stained by H & E and were evaluated by the pathologist.

Also, for evaluation of Bcl-2 protein Avidin-biotin (Dakocytomation kit, M 0887) immunohistochemistry staining was performed on tissue samples. Percentage and type of cells in which Bcl-2 protein is expressed was detected by a pathologist. For the comparison of results with those of normal group, 13 pathological blocks from healthy people prepared in the medical examiner's office (after obtaining written consent from their relatives), were used.

Slides were prepared from these samples according to the above-mentioned methods, were stained by H & E and Avidin-biotin

immunohistochemistry stainings and were evaluated by a pathologist.

The inclusion criteria for control samples were as follows:

All samples were taken within 48 hours after death from males with a mean age of 47± 4.3 yrs whose cause of death was head trauma without lung injury or cerebrovascular accidents. Also, according to their medical records, these patients had no history of cigarette smoking or any chronic or acute respiratory disease. In addition, lung specimens were reported normal by the pathologist after staining. On each slide stained by H & E, the percentage of inflammatory cells (neutrophil, lymphocyte, and macrophage) was determined by calculating the number of each type of cells in 5 microscopic fields at 400X magnification and their percentage to all inflammatory cells was determined. On slides stained immunohistochemically by Avidin-biotin, percentage and type of cells in which Bcl-2 protein had been expressed were determined and the results of the two groups were compared by using SPSS-13 software and student t-test. P-value less than 0.05 was considered significant.

RESULTS

According to the obtained results, in spite of the up-regulation of Bcl-2 protein in macrophages of victims ($p<0.001$), the number of macrophages in these specimens was significantly lower than that of healthy controls ($p<0.001$). Also, a significant increase was noted in the number of lymphocytes of victims compared to that of normal group ($p<0.001$). However, no significant difference was detected between the two groups neither in the Bcl-2 protein in lymphocytes nor in the number of neutrophils and Bcl-2 protein in these cells. But Bcl-2 protein in epithelial cells showed a significant increase in the lung specimens of victims compared to the normal group ($p<0.001$). Also, Bcl-2 protein in the victims' fibroblasts was significantly higher than that of the normal group ($p<0.05$).

Figures 1 and 3 demonstrate H & E staining and Table 1 shows the results obtained from evaluation of the stained slides and type of cells. Figures 2 and 4 demonstrate the Avidin-biotin staining and Table 2 shows the results obtained from evaluation of the stained slides.

Table 1. The mean and standard deviation of different types of cells in H & E staining of specimens

Type of cell		Mean ±SD	P-value
Macrophage	Normal group	60.55±14.24	P<0.001
	Study group	2.46±1.33	
Neutrophil	Normal group	13.88±4.85	P>0.05
	Study group	18.92±23.7	
Lymphocyte	Normal group	25.55±13.09	P<0.001
	Study group	77.84±23.79	

Table 2. The mean and standard deviation of Bcl-2 protein expression in different types of cells in the specimens

Type of cell		Mean percentage of Bcl-2 protein expression	P-value
Epithelial	Normal group	27.66±29.03	P<0.001
	Study group	88.46±19.51	
Macrophage	Normal group	47.22±14.38	P<0.001
	Study group	92.30±22.41	
Noutrophil	Normal group	85.55±11.02	P>0.05
	Study group	71.53±21.83	
Lymphocyte	Normal group	28.66±30.48	P>0.05
	Study group	42.30±23.50	
Fibroblast	Normal group	00.00	P<0.05
	Study group	24.00±21.16	

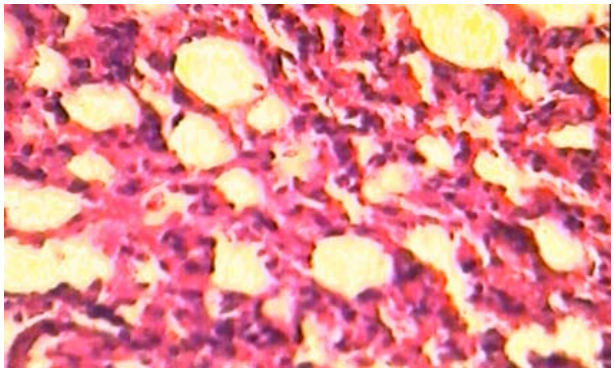

Figure 1. H & E staining of victims' lung specimens

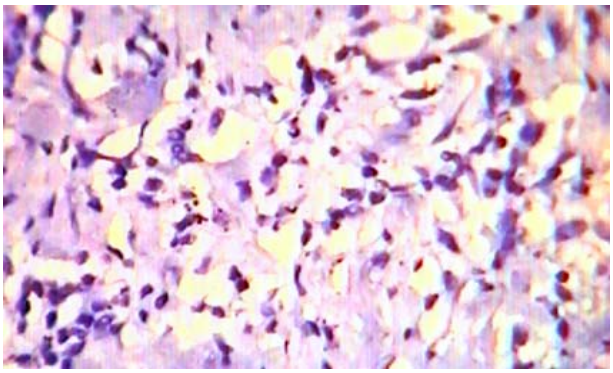

Figure 2. Bcl-2 immunohistochemistry staining of victims' lung specimens

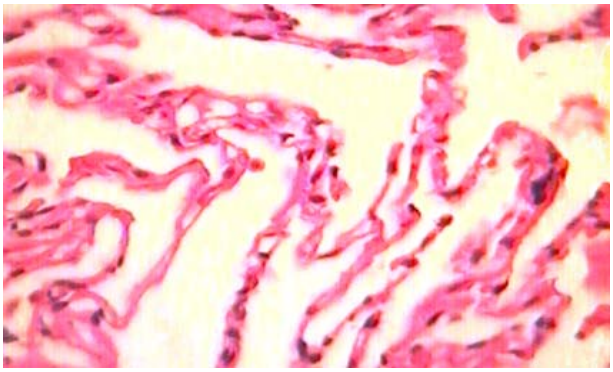

Figure 3. H & E staining of normal lung specimens

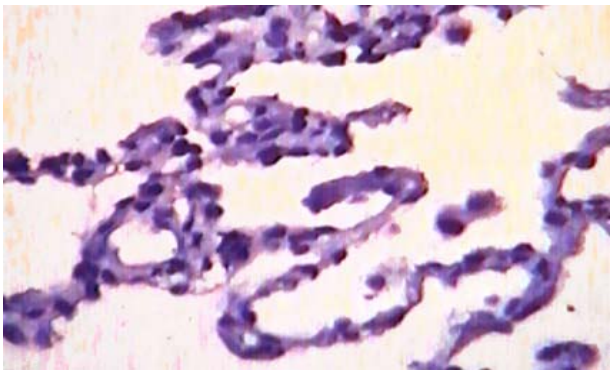

Figure 4. Bcl-2 immunohistochemistry staining of normal lung specimens

DISCUSSION

This was a study evaluating the expression of Bcl-2 gene in pulmonary fibrosis caused by sulfur mustard gas. Our study results indicated an increased number of lymphocytes and neutrophils in the victims compared to the controls. Also, number of macrophages was considerably decreased in these patients. Considering the results of this study and their comparison with other studies in the field of pulmonary fibrosis, changes in the percentage of different types of cells in lung specimens of victims was similar to those in IPF patients (23-25). Also, in a study conducted by Emad and Rezaian in 1999, cellular changes in bronchoalveolar lavage (BAL) specimens of sulfur mustard gas victims were similar to those of IPF patients. Therefore, IPF can be considered as a late pulmonary complication in these victims (11).

The main pathogenesis of pulmonary fibrosis was evaluated and approved during 1970-1980 indicating the role of inflammation in development of pulmonary fibrosis (26). In this process, lung injury due to an extrinsic factor results in inflammation followed by proliferation of fibroblasts and eventually pulmonary fibrosis. The increased number of neutrophils and lymphocytes in sulfur mustard gas victims in this study confirmed the role of inflammation in development of pulmonary fibrosis. This is important because it can be suggestive of the hypothesis of using anti-inflammatory drugs for prevention of this disease in initial stages of toxicity with sulfur mustard gas.

As we mentioned earlier, Bcl-2 protein which is resulted from the expression of Bcl-2 gene, belongs to the group of proteins regulating cell death (27) which extends cell lifespan by reducing apoptosis. Results obtained from avidin-biotin staining and anti-Bcl-2 protein monoclonal antibody of victims' lung specimens and its comparison with normal tissue indicated a significant up-regulation of Bcl-2 gene in fibroblasts of victims' lung specimens compared to the normal tissue. Fibroblasts are the main source of collagen production and factors responsible for pulmonary fibrosis (28, 29). In normal conditions, these cells have a temporary key role in resolving inflammation and tissue scarring and will be eliminated from the scene by the process of

apoptosis (30, 31).

At present, there is a definite evidence regarding the direct role of fibroblasts in development of pulmonary fibrosis (32) in a way that deranged apoptosis of fibroblasts and their extended lifespan result in higher collagen production and subsequent pulmonary fibrosis (33, 34). Up-regulation of Bcl-2 gene following exposure to SM can be the reason for decreased apoptosis of these cells and consequent increase in collagen production and eventually pulmonary fibrosis in these patients. However, this finding is different from the results of Tanaka's study regarding the expression of Bcl-2 gene in cultured lung fibroblasts (35). He reported that the expression of this gene was not different in normal lung fibroblasts and those of a fibrotic lung. However, considering the confirmed role of fibroblasts and their extended lifespan in development of pulmonary fibrosis, unchanged expression of Bcl-2 gene in fibroblasts of patients with pulmonary fibrosis can not be well justified.

Some studies are indicative of increased apoptosis in alveolar epithelial cells followed by inadequate alveolar epithelialization, destruction of alveolar wall and eventually a fibrotic tissue-repair response during the process of pulmonary fibrosis (36, 38). Also, increased proapoptotic and decreased anti-apoptotic proteins have been reported in IPF patients (39). However, Kazufumi and colleagues studied Bcl-2 gene expression in IPF patients and demonstrated that this gene had expressed in none of the epithelial cells of a fibrotic lung (40). Also, Plataki and colleagues in a similar study indicated a mild increase in the expression of this gene in epithelial cells of a fibrotic lung (39). In our study, Bcl-2 gene expression had been significantly increased in the lung epithelial cells of victims. This finding is different from the results of other studies regarding the Bcl-2 gene expression in the epithelial cells in pulmonary fibrosis due to other causes. Up-regulation of Bcl-2 gene in the lung epithelial cells of victims may be due to the increased expression of this gene in type II pneumocysts and consequent extension in lifespan of these cells followed by pulmonary fibrosis because type II epithelial cells are responsible for production of fibrogenic cytokines and growth factors and can promote fibrotic responses (38). Therefore, further studies on evaluation of the type of pneumocytes in which expression of Bcl-2 gene had been increased can provide more accurate data in this regard.

It is noteworthy that in macrophages in spite of significant increase in Bcl-2 protein (compared to normal tissue), number of these cells significantly decreased in lung specimens of victims. Considering this finding, a suggested hypothesis is that only the macrophages in which Bcl-2 gene had been expressed as an anti-apoptotic gene can survive and have function in the lung parenchyma of victims. Regarding the results of this study compared with other studies, sulfur mustard gas can be considered as a cause of IPF. Also, increased expression of Bcl-2 gene can be suggested as a possible mechanism in development of pulmonary complication. It is recommended that the effect of Bcl-2 inhibitors such as anti-Bcl-2 antibody be evaluated in the process of disease in future studies.

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