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The Role of Strain Variation in BAX and BCL-2 Expression in Murine Bleomycin-Induced Pulmonary Fibrosis

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Abstract: This study hypothesized that the expression of apoptosis-regulatory genes, such as BCL-2 and BAX may be affected by genetic variation in bleomycin-induced pulmonary fibrosis in C57BL/6 and NMRI mice. Pulmonary fibrosis induced by single intratracheal dose of bleomycin (3 U kg^{-1}). After 2 weeks, lung samples were analyzed for collagen deposition, pathological changes and expression of BCL-2 and BAX. The fibrotic lung changes were similar in both strains. The immunohistochemical assay using a biotin-streptavidin technique showed no significant difference in immunoreactivity for BCL-2 protein between the controls and bleomycin-treated C57BL/6 mice. However, in NMRI mice, the expression of BCL-2 was significantly ($p<0.05$) upregulated in myofibroblasts and neutrophils. The expression of BAX protein was significantly ($p<0.05$) upregulated in alveolar epithelial cells of both strains and downregulated in myofibroblasts and lymphocytes of the lung tissues of C57BL/6 mice and also in lymphocytes of NMRI mice at 2 weeks after bleomycin instillation. These results confirm the role of BCL-2 and BAX proteins in the pathogenesis of pulmonary fibrosis and suggest that the expression of apoptotic regulatory genes may be specific in different cell types in various strains.

Key words: BCL-2, BAX, bleomycin, pulmonary fibrosis, mice

INTRODUCTION

Apoptosis may play an important causal role in the pathogenesis of pulmonary fibrosis. A growing body of evidence suggests that apoptosis of alveolar epithelial cells is sufficient to initiate a fibrotic response (Kuwano *et al*., 2001; Selman *et al*., 2001; Uhal, 2002). One of the most important regulators of apoptosis is the BCL-2 family of proteins (Miyashita *et al*., 1994; Oltvai *et al*., 1993). BCL-2 family members have important role in the alveolar epithelial cell death and development of pulmonary fibrosis from bleomycin (Lee *et al*., 2005; Budinger *et al*., 2006). These proteins also have key roles in the pathogenesis of inflammation, fibrosis and apoptosis from transforming growth factor-beta which is a critical factor in interstitial diseases (Kang *et al*., 2007). BCL-2 protein is an intracellular membrane-associated protein, which localizes to the cytoplasmic face of the mitochondrial outer membrane, endoplasmic reticulum and perinuclear membranes and promotes cell survival by inhibiting apoptosis (Adams and Cory, 1998; Kinloch *et al*., 1999).

BAX gene, a member of the same family, encodes a membrane and two forms of cytosolic protein whose over expression accelerates apoptotic death and also antagonizes the death repressor activity of BCL-2. The ratio of BAX to BCL-2 determines susceptibility of a cell to apoptosis following an apoptotic stimulus (Oltvai *et al*., 1993).

Bleomycin-induced pulmonary fibrosis has been widely used as an experimental model for studying the cellular and molecular mechanisms of interstitial lung fibrosis (Hoshino *et al*., 2003; Lazo *et al*., 1990). It is well known that the extent of pulmonary fibrosis is strain-dependent after exposure to various stimuli such as bleomycin however, the mechanisms of strain variation

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and the genetic basis of susceptibility to pulmonary fibrosis are largely unknown (Bonniaud *et al*., 2004; Harrison and Lazo, 1988; Lemay and Haston, 2005). Murine strain variation has been reported in acute pulmonary damage and poly (ADP-ribose) polymerase activity from bleomycin (Hoyt and Lazo, 1992). Analysis of cytokine mRNA levels has been associated with strain variation in pulmonary fibrosis from bleomycin (Baecher-Allan and Barth, 1993). There are also limited studies about the functional genomics and genetic susceptibility to lung apoptosis. In the present study, NMRI mice were compared with C57BL/6 mice, a sensitive strain to bleomycin-induced pulmonary fibrosis, by biochemical analysis of collagen deposition and morphological examination of pathological lung changes. Increased apoptosis has been previously identified in bleomycin-induced pulmonary fibrosis in mice (Hagimoto *et al*., 1997). For better understanding the functional genomic of pulmonary apoptosis, we also hypothesized that the expression of apoptosis-regulatory genes, such as BCL-2 and BAX may be affected by genetic variation in two different strains of mice.

MATERIALS AND METHODS

Animals: Female C57BL/6 mice and NMRI mice, 8-10 weeks of age and 25-30 g body weight, obtained from Pasteur Institute (Tehran, Iran) were used. Animals were housed in standard laboratory cages, a 12 h light/dark cycle and 20±2°C was maintained and mice had access to water and rodent laboratory chow *ad libitum*. The mice were acclimated to the laboratory conditions for at least 1 week prior to the experiments. All experiments were approved by the Animal Research Committee of the Isfahan University of Medical Sciences and carried out according to the internationally accepted guidelines of the care and use of laboratory animals. This study was conducted from June 2005 to March 2007.

Bleomycin model of pulmonary fibrosis: Animals were anesthetized by intraperitoneal injection of 75 mg kg^{-1} ketamine (Rotexmedica Co., Germany). The trachea was exposed and bleomycin hydrochloride (Nippon Kayaku Co., Japan) was instilled at a single dose of 3 U kg^{-1} in 50 μL of sterile saline. Control mice received an equal volume of sterile saline intratracheally. Six animals were used in each control and experimental groups. After two weeks, animals were sacrificed with ketamine overdose. Lungs were removed and then weighed. The left lobe of lung tissue from each mouse was taken for biochemical analysis of collagen content. The right lung was processed for histological examination. The right lung samples were perfused with 10% neutral-buffered formalin via the trachea at 20 cm of H_2O constant pressure and kept in formalin solution for 24 h and then embedded in paraffin. The 4 μm thick paraffin sections were used for immunohistochemical, haematoxylin-eosin (H and E) and Masson's trichrome staining.

Biochemical analysis: For quantitation of collagen in the lungs, hydroxyproline content was measured by colorimetric method as described by Woessner (1961). L-hydroxyproline and all other reagents with analytical grade for biochemical assays were obtained either from Merck (Germany) or Sigma Chemical Co. (England).

In brief, lungs were removed at 2 weeks post bleomycin instillation. After homogenization and processing according to the method, absorbance was measured at 550 nm with a Unico UV-2100 spectrophotometer (United product, USA). A series of 0-10 μg mL^{-1} hydroxyproline concentrations were used to establish a standard curve.

Immunohistochemistry (IHC) for BCL-2 and BAX: IHC for BCL-2 (rabbit anti-mouse polyclonal antibody, A18, 1:100, Delta Biolabs, CA) and BAX (rabbit anti-mouse polyclonal antibody, 1:100, Dako, CA) were performed on formalin-fixed and paraffin-embedded tissues using a biotin-streptavidin technique (Giorno, 1984). Deparaffinized lung sections were placed in the antigen retrieval solution containing 10 mmol L^{-1} citrate buffer (pH 6) and heated in a microwave oven (700 W) for 10 min. Tissue endogenous peroxidase activity was quenched by incubation with 3% hydrogen peroxide in methanol. Nonspecific antibody binding was blocked with a solution of normal rabbit serum (Dako). Then, the slides were incubated for 30 min at room temperature with BCL-2 and BAX antibodies. After rinsing with PBS solution, the sections were incubated with biotinylated goat anti-rabbit IgG (1:200, Dako) for 10 min and were labeled by streptavidin-peroxidase complex (1:200, Dako) for 5 min. Diaminobenzidine was used as a substrate chromogen. Finally, slides were counter stained with hematoxylin and were mounted and cover slipped. The normal lymph node of the lung tissue was used as an internal positive control for BCL-2 and sections of normal breast tissue were used as a positive control for BAX. For negative controls, the primary antibodies were replaced by rabbit serum (Guinee *et al*., 1997).

The intensity of immunohistochemical staining was graded semiquantitatively as follows: grade 0 = no staining present or less than 10% of the cells are positive; grade 1 = 10% of the cells are positive; grade 2 = more

than 10% and less than 50% of the cells are positive and grade 3 = more than 50% of the cells are positive (Plataki *et al*., 2005).

Statistical analysis: Data were presented as Mean±SEM (Standard Error of Mean) and statistical analysis was made by one-way ANOVA followed by Dunnett analysis. For grading analysis, the data were presented as semiquantitative grades and evaluated by the nonparametric Kruskal-Wallis method followed by Mann-Whitney tests. p value<0.05 was considered statistically significant.

RESULTS

Hydroxyproline content of lung: Figure 1 shows the results of lung collagen measurement by hydroxyproline assay in two strains of mice, 2 weeks after bleomycin instillation (3 U kg^{-1}). There was a significant increase in collagen content by 47 and 45% in C57BL/6 and NMRI mice, respectively.

Histological findings: Morphological examination of lung tissues in C57BL/6 and NMRI mice was carried out at 2 weeks after bleomycin or saline instillations. Representative histological findings are shown in Fig. 2. Two weeks after exposure to bleomycin, there was thickening of the alveolar walls, infiltration of lymphocytes and macrophages into the interstitium and increased amount of collagenous fibers in the interstitial areas in both strain of mice (Fig. 2a, b). There was no

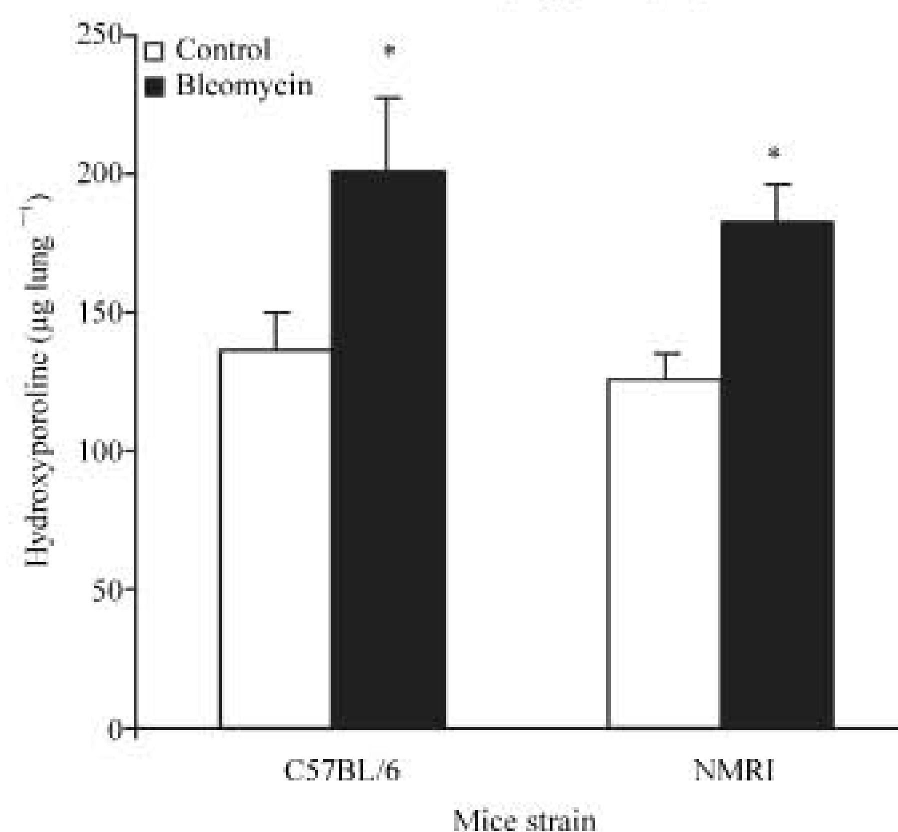

Fig. 1: Hydroxyproline concentration in lung tissue from C57BL/6 and NMR mice. Hydroxyproline was measured at 2 weeks after bleomycin (3 U kg^{-1}) or saline (control) instillation. Values are Means±SEM from 6 animals at each time point. *, p<0.05 versus corresponding control

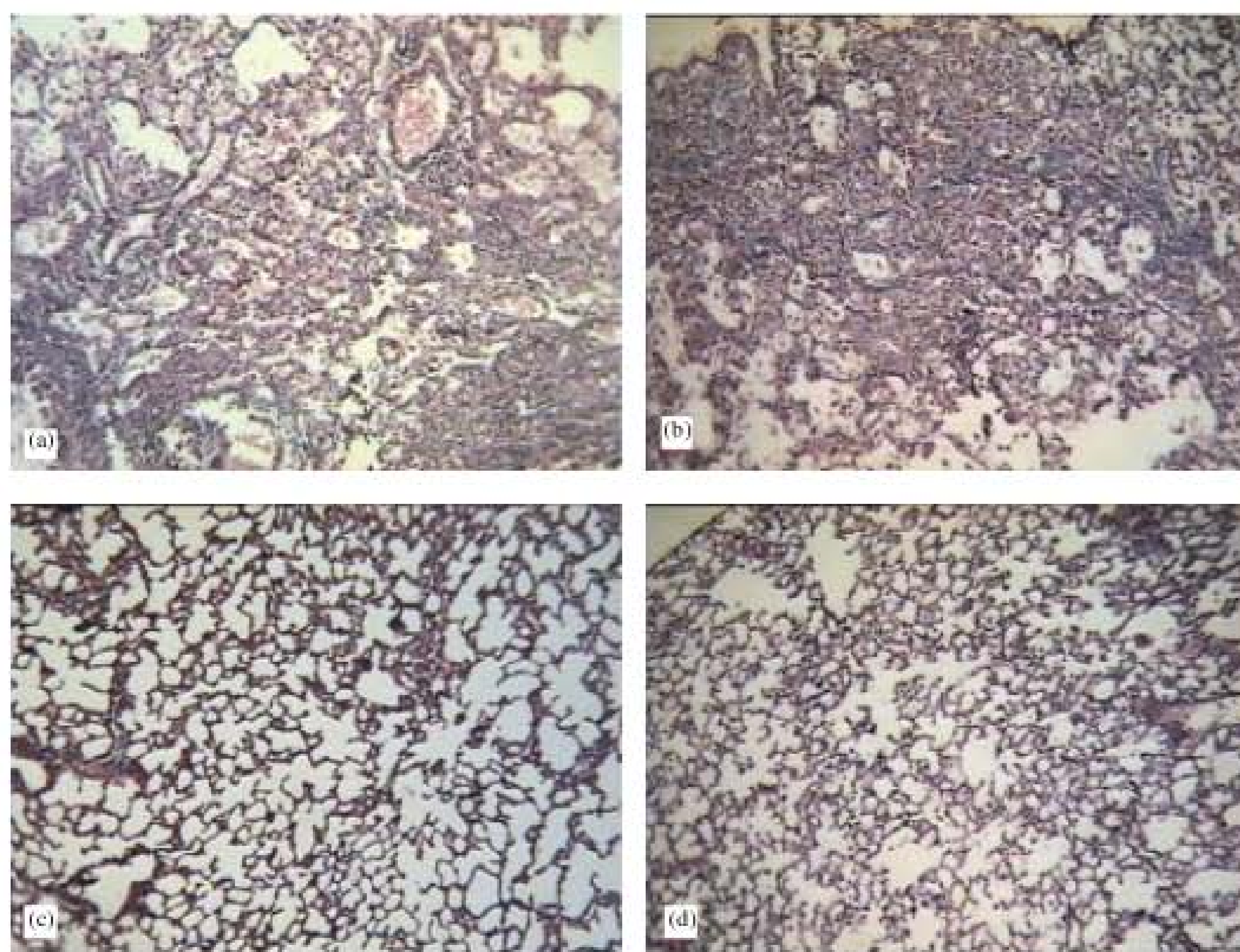

Fig. 2: Masson's trichrome histologic sections of lung tissues of C57BL/6 and NMRI mice (2a and b, respectively). At 2 weeks after bleomycin instillation, thickening of the alveolar walls, increase in the cellularity of alveolar septa and collagenous fibers were observed. There was no pathological change in the lungs parenchyma of saline-treated C57BL/6 and NMRI mice (2c and d, respectively). Magnification: x100

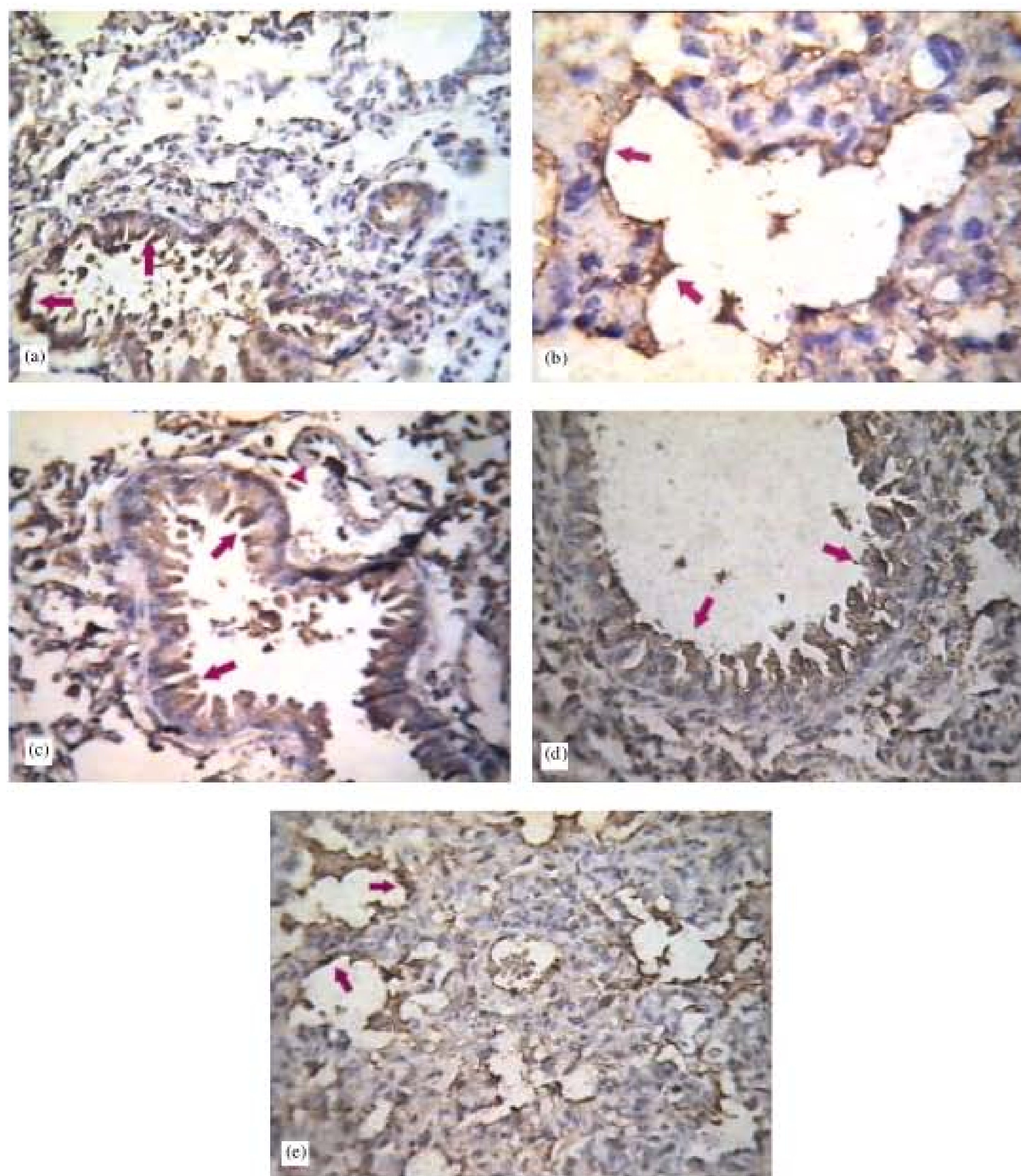

Fig. 3: Immunohistochemical localization of BCL-2 and BAX protein. The positive signals for BCL-2 are observed in the bronchiolar and alveolar epithelial cells of C57BL/6 (arrows in 3a and b, respectively) and in the bronchiolar epithelial cells and fibroblasts of NMRI mice (arrows and arrow-head in 3c, respectively). The positive signals for BAX are observed in the bronchiolar epithelial cells of C57BL/6 (arrows in 3d) and in the alveolar epithelial cells of NMRI mice (arrows in 3e). Magnifications: x1000 (b), x400 (a, c, d and e)

pathological change in the lungs obtained from the control animals (Fig. 2c, d).

IHC for BCL-2 and BAX: IHC for BCL-2 was positive in the bronchiolar (ciliated) and alveolar epithelial cells, macrophages and interstitial myofibroblasts in normal lung tissues as well as in the lungs of bleomycin-instilled C57BL/6 mice 2 weeks after instillation. In NMRI mice, BCL-2 protein was detected in bronchiolar and alveolar epithelial cells, macrophages, lymphocytes, neutrophils and interstitial myofibroblasts at 2 weeks after bleomycin instillation but not in neutrophils and interstitial myofibroblasts of the controls (Fig. 3a-c).

BAX protein was positive in bronchiolar and alveolar epithelial cells, macrophages, lymphocytes and interstitial myofibroblasts of the normal lung tissues of C57BL/6 and NMRI mice but not in lymphocytes of the bleomycin-instilled mice (Fig. 3d, e).

The semiquantitative results of IHC staining for BCL-2 and BAX are shown in Table 1 and 2. In C57BL/6 mice, there was no significant difference in immunoreactivity for BCL-2 protein between the controls and bleomycin-treated mice. However, in NMRI mice, the expression of BCL-2 was significantly ($p<0.05$) upregulated in myofibroblasts and neutrophils after bleomycin instillation. The expression of BAX protein was

Table 1: Expression of BCL-2 in lung tissues from control and bleomycin-treated mice

	C57BL/6 mice				NMR I mice			
	Control		Bleomycin		Control		Bleomycin	
Type of cells	F	G	F	G	F	G	F	G
Bronchiolar epithelial cells	57.5±8.0	2	47.0±2.5	2	14.0±4.5	1	10.5±2.0	1
Alveolar epithelial cells	55.5±6.0	2	52.5±4.0	2	50.5±1.0	2	45.0±4.0	2
Interstitial myofibroblasts	48.0±2.5	2	36.5±3.0	2	8.0±1.5	0	20.5±3.0*	2
Alveolar macrophages	36.0±5.0	2	37.0±4.0	2	76.0±6.0	3	78.5±1.5	3
Lymphocytes	7.5±2.0	0	6.5±1.0	0	55.0±5.0	3	57.5±4.0	3
Neutrophils	2.5±1.0	0	3.5±2.0	0	6.0±1.5	0	12.0±2.0*	1

F: Frequency expressed as percentage of immunoreactive cells; G: Staining grade expressed as: Grade 0: No staining present or less than 10% of the cells are positive; Grade 1: 10% of the cells are positive; Grade 2: More than 10% and less 50% of the cells are positive and Grade 3: More than 50% of the cells are positive. Data is presented as Mean±SEM for frequency and as the median grade of the immunoreactivity for 6 mice, * p<0.05 versus corresponding control

Table 2: Expression of BAX in lung tissues from control and bleomycin-treated mice

	C57BL/6 mice				NMRI mice			
	Control		Bleomycin		Control		Bleomycin	
Type of cells	F	G	F	G	F	G	F	G
Bronchiolar epithelial cells	49.5±10	2	27.0±5.0	2	36.3±6.5	2	20.6±3.5	2
Alveolar epithelial cells	34.0±4.5	2	64.5±3.0*	3	32.0±2.5	2	60.0±3.5*	3
Interstitial myofibroblasts	37.0±7.5	2	10.5±1.0*	1	8.0±2.5	1	9.5±1.5	1
Alveolar macrophages	47.2±2.0	2	45.0±2.5	2	31.8±4.5	2	34.5±4.0	2
Lymphocytes	11.0±1.0	1	5.0±1.5*	0	10.0±0.5	1	5.5±1.5*	0
Neutrophils	1.0±1.5	0	2.3±2.0	0	3.5±0.5	0	2.5±2.5	0

F: Frequency expressed as percentage of immunoreactive cells; G: Staining grade expressed as: Grade 0: No staining present or less than 10% of the cells are positive; Grade 1: 10% of the cells are positive; Grade 2: More than 10% and less 50% of the cells are positive and Grade 3: More than 50% of the cells are positive. Data is presented as Mean±SEM for frequency and as the median grade of the immunoreactivity for 6 mice, * p<0.05 versus corresponding control

significantly (p<0.05) upregulated in alveolar epithelial cells of both strains and downregulated in myofibroblasts and lymphocytes of the lung tissues of C57BL/6 mice and also in lymphocytes of NMRI mice at 2 weeks after bleomycin instillation.

DISCUSSION

Present findings showed that NMRI mice developed lung fibrosis at 2 weeks after bleomycin instillation and there was a good correlation between the lung fibrotic changes in NMRI and that of C57BL/6 mice.

In this study, immunoreactivity to BCL-2 and BAX was determined in different cells including lymphocytes, macrophages, neutrophils, alveolar epithelial cells and myofibroblasts, which their interactions are associated with development of interstitial fibrosis (Mason *et al*., 1999). We found the expression of BCL-2 in the alveolar and bronchiolar epithelial cells in normal and fibrotic lungs. Present results were similar to the findings of Plataki *et al*. (2005) who detected BCL-2 in alveolar epithelial cells in Idiopathic Pulmonary Fibrosis (IPF). Whereas, Kuwano *et al*. (2000) reported the absence of BCL-2 in alveolar epithelial cells of the ICR mice and Kazufumi *et al*. (1997) did not detected BCL-2 in epithelial cells of patients with IPF. The expression of BAX was upregulated in alveolar epithelial cells in fibrotic lungs and the BAX/BCL-2 ratio in these cells showed an increase in their susceptibility to apoptosis, which subsequently leads to pulmonary fibrosis.

An interesting finding in the results was upregulation of BCL-2 expression in myofibroblasts of bleomycin-induced fibrotic lungs in NMRI mice and downregulation of BAX expression in these cells in C57BL/6 mice. Fibroblasts and myofibroblasts, synthesizing the interstitial matrix, have critical role in the fibrotic transformation of the lungs. Present findings suggest that excessive proliferation and reduced apoptosis of these cells in fibrotic changes may be caused at least partially by various regulations of BCL-2 and BAX proteins; it means higher levels of BCL-2 in NMRI mice and lower levels of BAX in C57BL/6 mice.

Another interesting difference between the control and bleomycin groups was reduction in susceptibility of lymphocytes to apoptosis by downregulation of BAX expression in two strains of mice. However, there was significant difference in the percentage of BCL-2 expression in lymphocytes in NMRI and C57BL/6 mice. Also, there was a difference between two strains of mice on the expression of BCL-2 in neutrophils. In NMRI mice, there was upregulation of BCL-2 expression in neutrophils of blemycin treated group. These results suggest the protection of lymphocytes and neutrophils

from cell death in fibrotic changes. Mermigkis *et al*. (2001) also reported the over expression of BCL-2 protein in the neutrophils and eosinophils but not in the lymphocytes of the bronchoalveolar lavage fluid (BALF) of patients with IPF. In another study, Kuwano *et al*. (2000) reported that these changes possibly induced by upregulation of BCLX(L) in lymphocytes and macrophages. T-cells may play a role in the pathogenesis of pulmonary fibrosis, however, different subtypes of lymphocytes which infiltrate the injured lung may have diverse role in pulmonary fibrotic changes (Izbicki *et al*., 2002). Neutrophils may be responsible for the initiation of the inflammatory response in lung injury (Izbicki *et al*., 2002). Apoptosis of neutrophils, infiltrating leukocytes and mesenchymal cells have been implicated in the resolution of inflammation and attenuation of fibroblast proliferation (Kuwano *et al*., 2001; Razzaque *et al*., 2002). Present results suggest some differences in inflammatory response between two strains. However, further studies are required to define the time course of BCL-2 family proteins expression in different type cells of lung tissue in different strains of mice.

It is possible that different susceptibility to oxidant-induced lung injury may be responsible to some differences in BCL-2 and BAX expression in two strains of mice. Cellular levels of BCL-2 and BAX have been involved in the regulation of apoptosis damage induced by free radicals (Korsmeyer *et al*., 1995). Reactive oxygen species and DNA strand scission via., an oxidative process have been proposed as an important mechanism for lung toxicity from bleomycin (Hoshino *et al*., 2003). Leikauf *et al*. (2001) have studied the gene-environmental interactions, which control lung injury process. They found increased expression in genes associated with oxidative stress, anti-proteolytic function and repair of the extracellular matrix and suggested the strain variation in susceptibility to oxidant-induced lung injury.

In conclusion, present results confirmed the role of apoptosis in the pathogenesis of pulmonary fibrosis and suggest that the regulatory mechanisms of apoptosis may be specific in different cell types in various strains. Therefore, identifying the critical proapoptotic and antiapoptotic factors in specific cell types are essential for targeting key regulatory pathways in the treatment of pulmonary fibrosis.

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