Development and time-course of bleomycin-induced pulmonary fibrosis in NMRI mice

[*1]Jafarian-Dehkordi A., [2]Rabbani M., [2]Mir Mohammad Sadeghi H., [3]Afshar-Moghaddam N., [4]Alavi S.A., [3]Mahmoodi F, [2]Safaeian L.

[1]Isfahan Pharmaceutical Sciences Research Center, [2]Department of Pharmacology and Toxicology, Faculty of Pharmacy and Pharmaceutical Sciences, [3]Department of Pathology, Faculty of Medicine, Isfahan University of Medical Sciences, [4]Isfahan Science and Technology Town, East Sage Co., Isfahan, Iran.

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ABSTRACT

Bleomycin-induced pulmonary fibrosis is a widely used experimental model for human lung fibrosis. The severity of fibrosis varies among different strains of mice and investigation on different strains and finding the mechanisms of variation is important in understanding the pathogenesis of human lung fibrosis. In the present study, NMRI mice were used to investigate the severity and also time-course of bleomycin-induced pulmonary fibrosis in comparison with C57BL/6 mice. After single dose administration of intratracheal bleomycin, the fibrotic response was studied by biochemical measurement of collagen deposition and semiquantitative analysis of pathological lung changes. NMRI mice developed lung fibrosis from 1 to 4 week after bleomycin instillation, with significant increases in lung collagen content and significant morphological changes ($P < 0.05$). These findings indicate that NMRI mice might be suitable as an experimental model of bleomycin-induced lung fibrosis.

Keywords: Pulmonary fibrosis, Bleomycin, NMRI mice

INTRODUCTION

Bleomycin is an effective chemotherapeutic agent used for a variety of human malignancies (1). Unlike other cytotoxic drugs, bleomycin does not induce major myelosuppression or immuno-suppression, but repeated systemic or a high dose administration of bleomycin often leads to lung injury and pulmonary fibrosis (2). As bleomycin produces pulmonary fibrosis in different species of mammals easily, it is widely used as an experimental model of human pulmonary fibrosis (3, 4).

Some genetic predispositions have been shown to have an important role in individual susceptibility of humans to lung fibrosis (5). In animals, the severity of bleomycin-induced pulmonary fibrosis varies among several different murine strains of mice (6, 7). For example, C57BL/6 mice are high responders, DBA/2 and Swiss mice are intermediate responders, and BALB/c mice are low responders to development of pulmonary fibrosis induced by bleomycin (8). However, there are not sufficient and precise data on other strains of mice.

NMRI (Naval Medical Research Institute, USA) mouse is largely used as an animal model in many experimental fields as well as for investigation of pathogenesis of particles-induced lung fibrosis (9, 10), but to our knowledge there is no report on bleomycin-induced lung fibrosis in this strain of mice. Investigations have shown that pulmonary responses are strain-dependent and the type of compound used to induce fibrosis has great influence on severity and pathogenesis of lung fibrosis (6, 11). In this study, the severity and also time-course of intratracheal bleomycin-induced pulmonary fibrosis in NMRI mice was investigated.

MATERIALS AND METHODS

Chemicals

Bleomycin (Nippon Kayaku Co., Japan), Ketamine (Rotexmedica Co., Germany), and L-hydroxyproline (Merck, Germany) were used. All other analytical grade reagents for histology and biochemical assays were bought either from Merck (Germany) or Sigma Chemical Co. (England).

Animals

Female NMRI mice and C57BL/6 mice (25-30 g), obtained from Pasteur Institute (Tehran, Iran) were housed in standard laboratory cages and a 12h light/dark cycle was maintained while mice

Correspondence: jafarian@pharm.mui.ac.ir

had access to water and rodent laboratory chow ad libitum. Experiments were carried out according to the ethical guidelines of the care and use of laboratory animals (12).

Experimental design

Animals were tracheostomized under anesthesia by intraperitoneal injection of 75 mg/kg ketamine, and bleomycin was instilled at doses of 0.075, 0.15 and 0.5 U in 50 µl of sterile isotonic saline (8). For comparison with C57BL/6 mice, 0.075 U of bleomycin was instilled in this strain of mice. Control mice were given the same volume of sterile saline. Six animals were used in each control and experimental groups. Two weeks after bleomycin instillation, all groups of animal were sacrificed with ketamine overdose. For the time-course study, in separate experiments, 0.075 U bleomycin-treated mice were sacrificed at 1, 2, 3 and 4 weeks postinstillation (12). Lungs were removed, washed with saline, and then weighed. From each mouse, right lobe of lung tissue was taken for biochemical analysis of collagen content. The left lobe of lung was perfused with 10% formaldehyde via the trachea and kept in formaldehyde solution for histological examination.

Biochemical analysis

To estimate the amount of collagen in the lungs, hydroxyproline content was measured (13). In brief, lungs were removed at weeks 1, 2, 3 and 4 post bleomycin instillation. After homogenization of lungs in 2 ml of PBS (pH 7.4), 0.5 ml of each sample was digested in 1 ml of 6 N HCl for 8 h at 120°C. Then 50 µl of citrate/acetate buffer (5% citric acid, 7.24% sodium acetate, 3.4% sodium hydroxide, and 1.2% glacial acetic acid, pH 6.0) and 1 ml of chloramine-T solution (282 mg of chloramine-T, 2 ml of n-propanol, 2 ml of H_2O, and 16 ml of citrate/acetate buffer) were added to 50 µl of samples, and allowed to stand for 20 min at room temperature. After addition of 1 ml of Ehrlich's solution (2.5 g of 4-dimethylamino benzaldehyde, 9.3 ml of n-propanol, and 3.9 ml of 70% perchloric acid) to each sample, the samples were placed in a water bath at 65°C for 15 min. Samples were cooled for 10 min and their absorbance was measured at 550 nm with a Unico UV-2100 spectrophotometer (United product, USA). A concentration series of 0-10 µg/ml hydroxyproline were used to establish a standard curve.

Histological examinations

Lung tissue was fixed by 10% neutral formalin solution for paraffin-embedded samples and sectioned at approximately 4 µm thickness. Sections were dewaxed, rehydrated and stained either by hematoxylin-eosin (H&E) or by a Masson's trichrome. The slides were examined by light microscopy and photographed. Semi-quantitative morphological study of pathological changes in lung sections was carried out to assess the severity of pulmonary fibrosis and graded according to the described method (14). The grade of pulmonary fibrosis was scored in a blinded fashion on a scale of 0 to 8 by examination of 10 randomly chosen regions per sample at a magnification of ×100. Criteria for grading pulmonary fibrosis were as follows: grade 0 = normal tissue; grade 1 = minimal fibrous thickening of alveolar or bronchial walls; grade 3 = moderate thickening of walls without obvious damage to the lung architecture; grade 5 = increased fibrosis with definite damages to the lung structure and formation of fibrous bands or small fibrous masses; grade 7 = severe distortion of structure and large fibrous areas; grade 8 = total fibrous obliteration of the field. If there was any difficulty in making decision between two odd-numbered grades, the field was given the intervening even-numbered score (14).

Statistical analysis

Statistical analysis was made by Student's t-test and one-way ANOVA followed by Dunnett analysis. Fibrosis grade was analyzed by the non-parametric Kruskal-Wallis test. Data were presented as mean ± SEM unless otherwise stated. P value <0.05 was considered significant.

RESULTS

Hydroxyproline content of lung

To determine an appropriate dose for analysis of lung fibrosis in NMRI mice, total lung collagen was measured as hydroxyproline content after a single administration of 0.075, 0.15 and 0.5 U of bleomycin. Our findings showed that administration of bleomycin (0.075-0.5 U) significantly increased hydroxyproline level after two weeks (Figure 1). Since there was a significant increase in collagen content without mortality and because of similarity with dose that is used for C57BL/6 mice, time-course of intratracheal bleomycin-induced lung fibrosis was studied using 0.075 U bleomycin instillation.

Time-course study of hydroxyproline production in lung tissue of mice after bleomycin (0.075 U) treatment showed a significant increase in hydroxyproline after 2, 3 and 4 weeks post bleomycin instillation (Figure 2). Figure 3 shows the results of comparison of NMRI mice with C57BL/6 mice treated with 0.075 U bleomycin after two weeks.

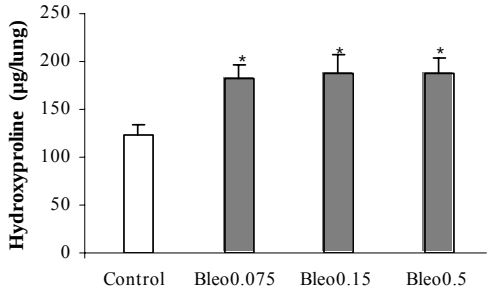

Figure 1. The effect of bleomycin on lung hydroxyproline content in NMRI mice. Two weeks after exposure to either intratracheal bleomycin (Bleo) (0.075-0.5 U) or saline (control), hydroxyproline content of lung tissue of mice was measured and normalized to micrograms per lung. Data are presented as mean ± SEM of n = 6; * $P < 0.05$ versus control.

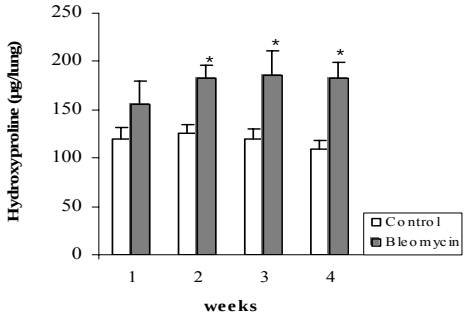

Figure 2. Time-course of hydroxyproline production in lung tissue of NMRI mice. Animals were exposed to either intratracheal bleomycin (0.075 U) or saline (control) and hydroxyproline content of lung tissue of mice was measured after 1, 2, 3 and 4 weeks and normalized to micrograms per lung. Data are presented as mean ± SEM of n = 6; * $P < 0.05$ versus control.

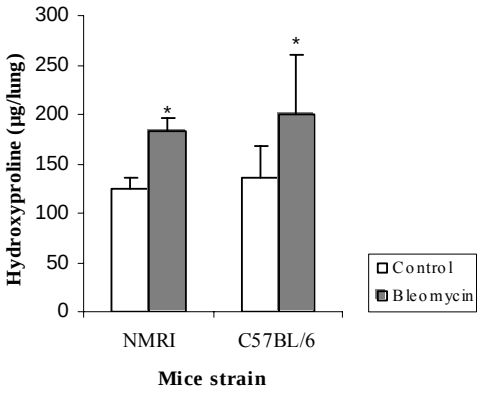

Figure 3. Comparison of hydroxyproline content in lung tissue of NMRI and C57BL/6 mice, two weeks after exposure to either intratracheal bleomycin (0.075 U) or saline (control). Data are presented as mean ± SEM of n = 6; * $P < 0.05$ versus control.

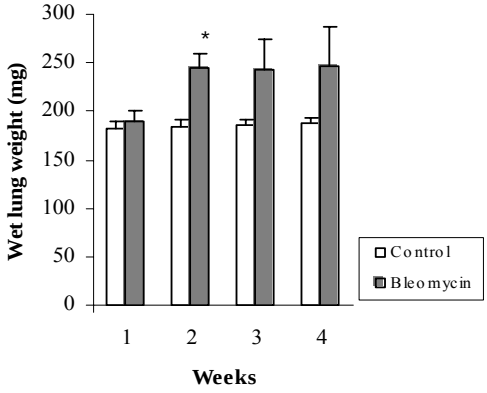

Figure 4. The effect of bleomycin on wet lung weight of NMRI mice. Animals were exposed to either intratracheal bleomycin (0.075 U) or saline (control). Data are presented as mean ± SEM of n = 6; * $P < 0.05$ versus control.

Lung weight

Total wet lung weight was measured as an indicator of lung inflammation. Bleomycin (0.075 U) significantly increased wet lung weight after 2 weeks ($P < 0.05$), but there was no significant changes after 3 and 4 weeks (Figure 4).

Histology

Morphological examination of lungs was carried out at 1, 2, 3 and 4 weeks after bleomycin (0.075 U) or saline instillations (Figure 5). Histological analysis showed that during the first week, there were slight pathological changes in lung. Focal areas of inflammation and slight infiltration of lymphocytes and macrophages were present and in some cases, minimal subcapsular collagen fibers were observed (Figure 5a) in contrast to lungs obtained from control animals (Figure 5e).

Two weeks after exposure to bleomycin, there was a moderate thickening of alveolar wall, increase in cellularity of alveolar septa and intra-alveolar fibrosis with small collagenous bands (Figure 5b). However, the severity of changes varied from slight to moderate.

After 3 and 4 weeks, there were slight to severe pathological changes (Figure 5c, 5d). In some animals, diffuse and definite damages to lung architecture, hyperplasia of type II pneumocytes, and increase in the amount of collagen in the interstitial areas as bands or fibrous masses were observed.

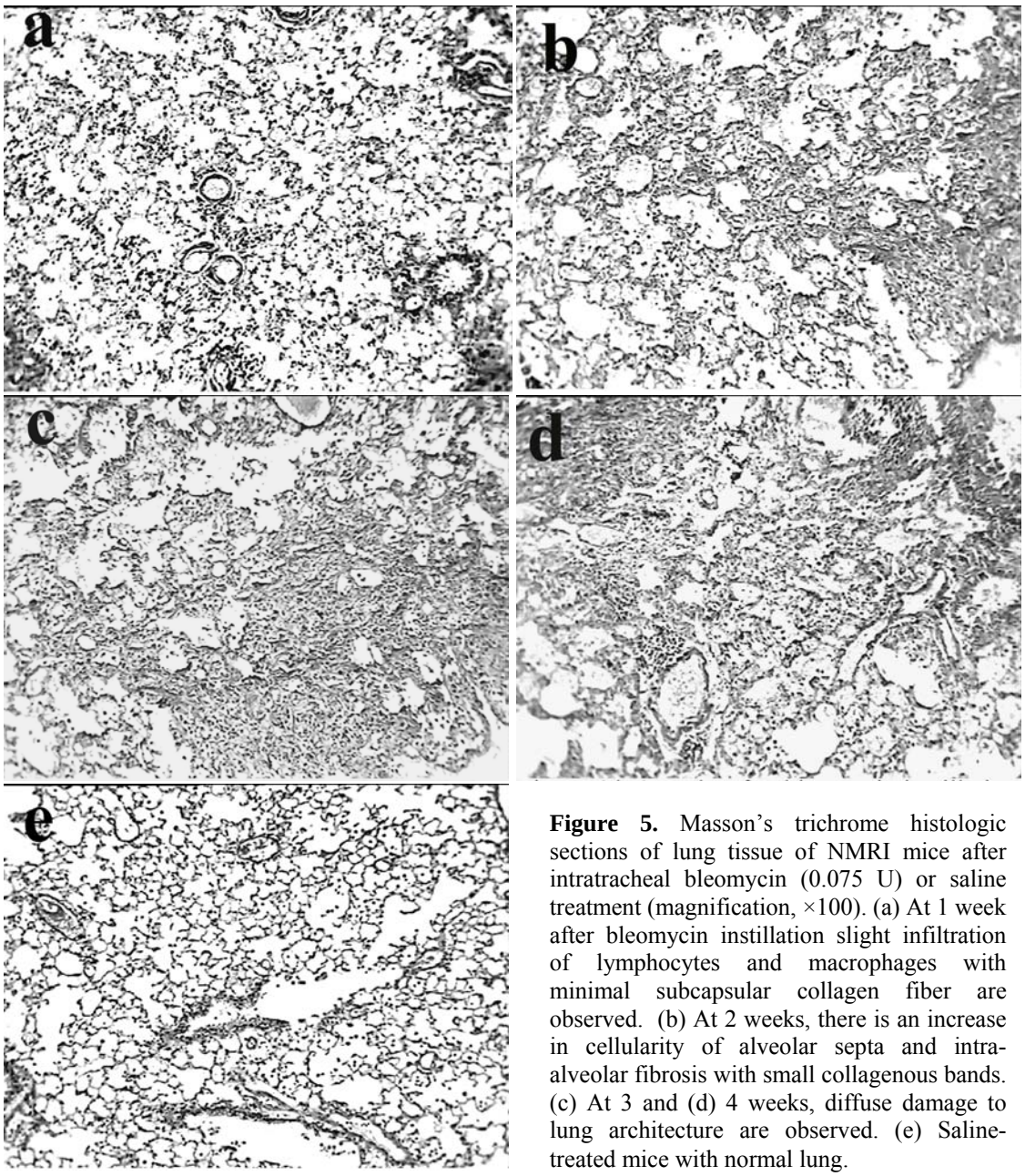

Figure 5. Masson's trichrome histologic sections of lung tissue of NMRI mice after intratracheal bleomycin (0.075 U) or saline treatment (magnification, ×100). (a) At 1 week after bleomycin instillation slight infiltration of lymphocytes and macrophages with minimal subcapsular collagen fiber are observed. (b) At 2 weeks, there is an increase in cellularity of alveolar septa and intra-alveolar fibrosis with small collagenous bands. (c) At 3 and (d) 4 weeks, diffuse damage to lung architecture are observed. (e) Saline-treated mice with normal lung.

Figure 6 represents the semiquantitative morphological changes of lung tissue. There were significant changes at 2, 3 and 4 weeks post bleomycin treatment ($P < 0.05$).

DISCUSSION

Intratracheal instillation of bleomycin is an experimental model of human interstitial lung fibrosis in laboratory animals for the study of the cellular and molecular mechanisms of pulmonary fibrosis, investigation of the new antifibrotic agents and also prevention of the side effects of bleomycin therapy (3).

It has been shown that in animal models, the extent of pulmonary fibrosis is strain-dependent however, the genetic basis of susceptibility to pulmonary fibrosis is largely unknown (6, 7, 15). The NMRI mouse, originally derived from Swiss mice (16) is a laboratory animal largely used in many fields of general biology as well as in pharmacology and toxicology (9). In toxicological studies, random bred of NMRI mice have been widely used, because it seems that the degree of variances between them and what would be expected in the human population are similar (17). NMRI mouse is an animal model extensively used

for investigation of pathogenesis of particles-induced lung fibrosis. After intratracheal instillation of three different types of particles (tungsten carbide, manganese dioxide, and crystalline silica) in NMRI mice, the pulmonary responses were different by the lack of inflammation, resolutive alveolitis and fibrosing alveolitis, respectively (11). Like bleomycin, the strain variability in sensitivity to silica has been known and DBA/2 mice developed the most severe fibrotic responses to silica particles (18).

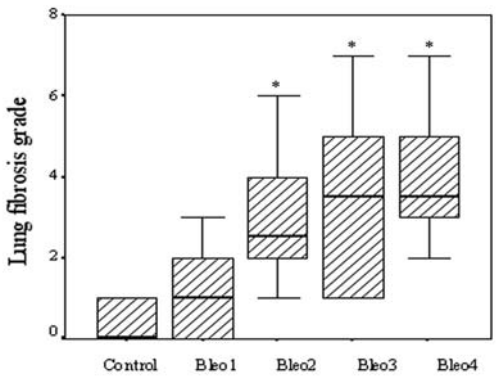

Figure 6. Semiquantitative scoring of lung fibrosis at 1, 2, 3 and 4 weeks after bleomycin (Bleo) instillation (0.075U). Thick lines represent the median of n = 6, boxes show the interquartile range and bars represent the maximum and minimum sample values. * $P< 0.05$ versus control.

The biological mechanism for these differences in response to fibrotic substances is not completely clear, but identification of these mechanisms is important to understand the pathogenesis of lung fibrosis for development of new drugs (15).

In this study, initially the effective dose of 0.075 U of bleomycin for induction of pulmonary fibrosis in NMRI mice was established. Although bleomycin significantly increased the level of hydroxyproline, a dose-dependent change in collagen deposition up to 0.15 U of bleomycin instillation was not observed. The mortality rate increased at doses greater than 0.5 U.

Pulmonary inflammation as noted by the increases in wet lung weight was only significant in mice at 2 weeks after treatment with bleomycin. It is important that in NMRI mice, the fibrotic responses were accompanied by a high expression of the anti-inflammatory and fibrotic cytokine IL-10 by silica-activated lung macrophages and anti-inflammatory treatment had no effect on the amplitude of lung fibrosis in silica-treated mice (10, 18). Taking these reported results in consideration, the role of inflammatory process in the pathogenesis of bleomycin-induced lung fibrosis in NMRI mice, remains to be determined.

In the time-course study, although there was a significant increase in collagen content at 2, 3 and 4 weeks after instillation of bleomycin in comparison with control, but it was not time-dependent after two weeks.

Morphological examinations by evaluation of alveolar wall thickness, damages to lung architecture, and formation of collagen fibers, revealed the development of fibrosis at 1 to 4 weeks after instillation of bleomycin. However, the severity of the lung changes varied from nearly normal to severe from mouse to mouse.

Good correlations were found by comparison of lung fibrotic changes in NMRI with C57BL/6 mice, as a sensitive strain to bleomycin-induced pulmonary fibrosis.

From these findings it may be concluded that a single intratracheal instillation of bleomycin in NMRI mice produces pulmonary fibrotic lesions making this strain of mice suitable as an experimental model for lung fibrosis. However further studies are required to define the mechanisms underlying variation among different strains of animals and to understand the precise role of genetic predisposition in lung fibrosis.

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