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Elastase modifies bleomycin-induced pulmonary fibrosis in mice

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ABSTRACT

Pulmonary fibrosis (PF) is characterized by excessive accumulation of collagen in the lungs. Emphysema is characterized by loss of the extracellular matrix (ECM) and alveolar enlargement. We studied the co-participation of elastase-induced mild emphysema in bleomycin-induced PF in mice by analyzing oxidative stress, inflammation and lung histology. C57BL/6 mice were divided into four groups: control; bleomycin (0.1 U/mouse); elastase (using porcine pancreatic elastase (PPE) + bleomycin (3 U/mouse 14 days before 0.1 U/mouse of bleomycin; PPE + B); elastase (3 U/mouse). Mice were humanely sacrificed 7, 14 and 21 days after treatment with bleomycin or vehicle. PF was observed 14 days and 21 days after bleomycin treatment but was observed after 14 days only in the PPE + B group. In the PPE + B group at 21 days, we observed many alveoli and alveolar septa with few PF areas. We also observed marked and progressive increases of collagens 7, 14 and 21 days after bleomycin treatment whereas, in the PPE + B group, collagen deposition was observed only at 14 days. There was a reduction in activities of the antioxidant enzymes superoxide dismutase ($p < 0.05$), catalase ($p < 0.01$) and glutathione peroxidase ($p < 0.01$) parallel with an increase in nitrite ($p < 0.01$) 21 days after bleomycin treatment compared with the control group. These endpoints were also reduced ($p < 0.05$, $p < 0.05$ and $p < 0.01$, respectively) and increased ($p < 0.01$) in the PPE + B group at 21 days compared with the control group. Interleukin (IL)-1β expression was upregulated ($p < 0.01$) whereas IL-6 was downregulated ($p < 0.05$) in the PPE + B group at 21 days compared with the control group. PF and emphysema did not coexist in our model of lung disease and despite increased levels of oxidative stress and inflammatory markers after combined stimulus (elastase and bleomycin) overall histology was improved to that of the nearest control group.

1. Introduction

Pulmonary fibrosis (PF) is a chronic disease characterized by extensive deposits of the extracellular matrix (ECM) that can impair the architecture and function of the lungs (Cavazza et al., 2010). Imbalance between the proliferation and apoptosis of fibroblasts results in increased synthesis/deposition of the ECM against degradation, leading to accumulation of collagen fibers and reduction of the respiratory surface (Todd et al., 2012). Development of PF may be rapid and, once installed, and patient survival is ≈3 years. PF can also display alternating long periods of stability as well as short episodes of exacerbation (Rafii et al., 2013). Susceptibility to PF appears to be related to the interaction between multiple factors, such as genetic predisposition (Santangelo et al., 2013), oxidative stress (Kliment and Oury, 2010) and the inflammatory response (Homer et al., 2011).

Bleomycin is a glycosylated peptide antibiotic produced by the bacterium *Streptomyces verticillus* and is used as an anticancer agent. However, there is a common reported side effect in that PF is induced (Moore et al., 2013) even though not all patients treated with bleomycin manifest it (Fernandez Perez, 2012). Several agents have been used to induce PF, such as irradiation (Epperly et al., 2003), silica (Shimbori et al., 2010) and bleomycin (Manoury et al., 2005). Participation of inflammation and oxidative stress in PF in animal models is well established (Moeller et al., 2008).

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Studies have demonstrated bleomycin-induced PF resistance in BALB/c mice that is due to the activity and expression of superoxide dismutase (SOD) (Santos-Silva et al., 2012).

Pulmonary emphysema is a chronic disease characterized by permanent destruction of the respiratory areas (*e.g.*, bronchioles, alveolar ducts, alveoli) that impair the architecture and function of the lungs (Bezerra et al., 2011). Our research team has developed two experimental models of emphysema that elicit changes similar to those observed in human emphysema: exposure to cigarette smoke (Valenca et al., 2004) and instillation of porcine pancreatic elastase (PPE) (Lanzetti et al., 2012). Participation of inflammation and oxidative stress in emphysema in animal models has been documented, and studies have demonstrated major participation of tumor necrosis factor (TNF)-α (Churg et al., 2002), matrix metalloproteinase (MMP)-12 (Valenca and Porto, 2008), nuclear factor-kappa B (Valenca et al., 2006) and oxidative stress (Rueff-Barroso et al., 2010).

Some scholars hypothesize that PF may be associated with a preexisting inflammatory disease (*e.g.*, pulmonary emphysema), or that emphysema and fibrosis develop in mice spontaneously, after exposure to cigarette smoke, or after single treatment with bleomycin (Bartalesi et al., 2005; Cavarra et al., 2001; Gardi et al., 1992; Lucattelli et al., 2005). PF can occur also in smokers exposed to a causative agent of fibrosis (Silva et al., 2008), so some individuals may develop pulmonary emphysema and PF simultaneously. We wished to produce an animal model of middle-aged smokers (using elastase) exposed to unsanitary conditions of toxic inhalants or treated with bleomycin. We also wanted to find evidence of emphysema lesions together with fibrotic areas, and to see if previous treatment with elastase reduced bleomycin-induced PF at 21 days.

2. Material and methods

2.1. Ethical approval of the study protocol

This study was carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals (National Institutes of Health, Bethesda, MD, USA). The protocol was approved by the Committee for Evaluation of Animal Use for Research of the Federal University of Rio de Janeiro (authorization number DFBCICB063). All experimental procedures were repeated twice.

2.2. Animals and experimental design

Seventy female C57BL/6 mice (6 weeks; 20–24 g) were purchased from the Veterinary Institute of Fluminense Federal University (Niterói, Brazil). Mice were housed (five per cage) in a controlled environment with a 12-h light–dark cycle (lights on at 7 am), an ambient temperature of 21 ± 2 °C and humidity of 40–60%. Animals had free access to water and food. Mice were allowed to acclimatize for two weeks before experimental procedures.

Fig. 1 shows the experimental design. Briefly, mice were anesthetized with ketamine hydrochloride (45 mg kg^{-1}; i.m.) and 2% xylazine (9 mg kg^{-1}; i.m.). Then, one group of mice received a bleomycin (i.t.) dose of 0.1 U in a final volume of 50 μL. Another group of mice a received PPE (i.n.) dose of 3 U in a final volume of 10 μL 14 days before the same mice received a bleomycin (i.t.) dose of 0.1 U in a final volume of 50 μL. A final group of mice received a PPE (i.n.) dose of 3 U in a final volume of 10 μL 14 days before the same mice received vehicle (i.t.) in a final volume of 50 μL. The control group received 10 μL (i.n.) and 50 μL (i.t.) of vehicle. Mice (n = 7/group) were sacrificed 7, 14 and 21 days after treatment with bleomycin or vehicle. All reagents were purchased from Sigma–Aldrich (Saint Louis, MO, USA).

2.3. Processing, histology and morphometry of tissue

One day after the end of experimentation, mice were sacrificed by cervical dislocation. Bronchoalveolar lavage fluid (BALF) was obtained from the left lungs of all mice. Briefly, the right lung was clamped, and a cannula inserted into the trachea. Airspaces from the left lungs were washed with buffered saline solution, and the flow-through (final volume, 1.2–1.5 mL) was maintained on ice. The right ventricles of all mice were perfused with physiologic (0.9%) saline to remove blood. Next, the right lungs of all animals were inflated with 4% phosphate-buffered formalin (pH 7.2) at 25 cm H_2O for 2 min, ligated and removed. Inflated lungs were fixed for 48 h before embedding in paraffin. To analyze the lung by morphometry, the sample design should take care of bias related to the orientation of the structures in the lung. Estimates of areas, lengths, and numbers can be obtained for lung specimens with isotropic uniform random (IUR) tissue section. The IUR sections were used to avoid influence of tissue anisotropy (Gundersen and Jensen, 1987). The right lung was sectioned perpendicularly to the lung base (apico–basal axis), giving origin of 2 halves with portions of the upper, middle, and base of the lung. Each paraffin tissue block contained the lung halves with the sectioned portions oriented to the cutting face of the block. Serial sectioning was performed, and sections with 30–100 μm of interval (IUR) were selected for staining and morphometry. Sagittal, 5-μm serial sections of right lungs were stained with hematoxylin & eosin (H&E) and Masson's trichrome for histological analyses. Left lungs were removed immediately and homogenized on ice with 1 mL of potassium phosphate (pH 7.3) using a homogenizer (NT136; Novatécnica Piracicaba, Brazil) and centrifuged at 600 × g for 10 min at room temperature. Supernatants, adjusted to a final volume of 1.5 mL with potassium phosphate, were stored at −20 °C until biochemical analyses. Total protein content in lung homogenates was determined by the Bradford method.

To obtain uniform and proportionate lung samples, 10 fields (five non-overlapping fields in two IUR sections; ×20 objective lens) were analyzed in upper, middle, and base of the lung using a video microscope (DML30; Leica, Wetzlar, Germany). The reference volume was estimated by point counting using the test points system (PT) by a M42 test-system superimposed on the monitor screen (Weibel et al., 1966). The points that made contact with alveolar septa (PP) and collagens were counted to estimate the volume densities (Vv) of the structures (V = PP/PT) in sections stained with H&E and Masson. Alveolar enlargement (emphysema) and fibrosis were quantified by measuring the mean linear intercept (Lm, mean alveolar diameter) in micrometers. Briefly, this method involves determination of the number of times the gas-exchange structures in the parenchyma intersect a series of grid lines. Lm was obtained using the equation:

$$\mathrm{Lm} = \frac{\mathrm{Ltot}}{\mathrm{Li}}$$

where Ltot is the total length of the lines in the microscopic field, and Li is the number of intercepts of alveolar structures with the lines of the reticulum. To that end, 10 fields of each slide were counted and observed at a magnification of ×200 through a reticulum attached to the monitor. General morphometry was not performed in bronchi areas, but only in lung parenchyma (respiratory area). The degree of parenchymal destruction was determined additionally by destructive index analysis by using a transparent sheet with 50 counting points (Saetta et al., 1985). The sheet was laid on an A4-size print, on which the microscopic images from the stained sections were projected using a video microscope (DML30;