Acta Histochemica xxx (2017) xxx–xxx

Contents lists available at ScienceDirect

Acta Histochemica

journal homepage: www.elsevier.de/acthis

Comparative study of two models of combined pulmonary fibrosis and emphysema in mice

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ARTICLE INFO

Article history:
Received 1 August 2016
Received in revised form
31 December 2016
Accepted 23 January 2017
Available online xxx

Keywords:
Combined pulmonary fibrosis and emphysema
MHV-68
Mice model

ABSTRACT

Combined pulmonary fibrosis and emphysema (CPFE) is an "umbrella term" encompassing emphysema and pulmonary fibrosis, but its pathogenesis is not known. We established two models of CPFE in mice using tracheal instillation with bleomycin (BLM) or murine gammaherpesvirus 68 (MHV-68). Experimental mice were divided randomly into four groups: A (normal control, n = 6), B (emphysema, n = 6), C (emphysema + MHV-68, n = 24), D (emphysema + BLM, n = 6). Group C was subdivided into four groups: C1 (sacrificed on day 367, 7 days after tracheal instillation of MHV-68); C2 (day 374; 14 days); C3 (day 381; 21 days); C4 (day 388; 28 days). Conspicuous emphysema and interstitial fibrosis were observed in BLM and MHV-68 CPFE mouse models. However, BLM induced diffuse pulmonary interstitial fibrosis with severely diffuse pulmonary inflammation; MHV-68 induced relatively modest inflammation and fibrosis, and the inflammation and fibrosis were not diffuse, but instead around bronchioles. Inflammation and fibrosis were detectable in the day-7 subgroup and reached a peak in the day-28 subgroup in the emphysema + MHV-68 group. Levels of macrophage chemoattractant protein-1, macrophage inflammatory protein-1α, interleukin-13, and transforming growth factor-β1 in bronchoalveolar lavage fluid were increased significantly in both models. Percentage of apoptotic type-2 lung epithelial cells was significantly higher; however, all four types of cytokine and number of macrophages were significantly lower in the emphysema + MHV-68 group compared with the emphysema + BLM group. The different changes in pathology between BLM and MHV-68 mice models demonstrated different pathology subtypes of CPFE: macrophage infiltration and apoptosis of type-II lung epithelial cells increased with increasing pathology score for pulmonary fibrosis.

1. Introduction

Pulmonary fibrosis (PF) is characterized by accumulation of inflammatory cells, a complex network of immunomodulatory cytokines and growth factors, and expansion of the population of mesenchymal cells. These events result in deposition of proteins from the extracellular matrix. In contrast, emphysema results in reduced elastic recoil in the lungs and premature closure of the airways. Consequently, emphysema is characterized by reductions in the indices of forced expiratory flow, with a reduction in the ratio of forced expiratory volume in one second to forced vital capacity.

Emphysema and PF are defined by distinct clinical, functional, imaging and pathologic characteristics. However, a growing body of evidence supports the existence of "combined pulmonary fibrosis and emphysema" (CPFE). Lung volumes and spirometry are relatively preserved in patients with CPFE, but they can experience marked reductions in diffusing capacity. In patients with CPFE, a common factor that has been described is a history of current or past use of tobacco (Cottin, 2013; Portillo and Morera, 2012; He et al., 2014; Papiris et al., 2013; Jankowich and Rounds, 2012).

Abbreviations: AI, apoptosis index; BLM, bleomycin; BALF, bronchoalveolar lavage fluid; CPFE, Combined pulmonary fibrosis and emphysema; DI, destructive index; EBV, Epstein Barr Virus; HE, hematoxylin and eosin; MLI, mean linear intercept; MCP-1/CCL2, macrophage chemoattractant protein-1; MIP-1α/CCL3, Macrophage inflammatory proteins-1α; IL-13, Interleukin-13; TUNEL, terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling; TFG-β1, transforming growth factor-β1.

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http://dx.doi.org/10.1016/j.acthis.2017.01.007
0065-1281/

Clinical features of CPFE are well known, but the mechanisms leading to CPFE are not clear. Therefore, an animal model of CPFE must be established to study CPFE pathogenesis.

Several clinical studies have demonstrated the relationship between infection with the Epstein–Barr virus (EBV), inflammation, and PF (Bossink et al., 2008; McManus et al., 2008; Stewart et al., 1999; Williams, 2014). In the present study, we sought to develop a murine model of CPFE by tracheal instillation of murine gammaherpesvirus 68 (MHV-68), a virus closely related to the EBV. We also used another method to create a CPFE model in mice by employing bleomycin (BLM). Then, we compared the results elicited using these two models.

2. Materials and methods

2.1. Experimental animals and design

The Ethics Committee of Tongji Medical College within the Huazhong University of Science and Technology (Wuhan, China) approved the study protocol, which was in accordance with the Helsinki Declaration.

Forty-two male, specific pathogen-free C57BL/6 mice (6–8 weeks) were purchased from the Center of Experimental Animals (Tongji Medical College) Mice were divided randomly into four groups: A (normal control; n = 6); B (emphysema; passive cigarette smoking for 1 h twice a day for 13 months; 0.1 mL phosphate-buffered saline (PBS) by tracheal instillation on day 361; n = 6); C (emphysema + MHV-68; 1×10^5 plaque-forming units of MHV-68 in 0.1 mL PBS by tracheal instillation on day 361; n = 24); D (emphysema + BLM; 2 mg/kg BLM in 0.1 mL PBS by tracheal instillation on day 361; n = 6). To establish an emphysema model, mice underwent passive smoking with filtered cigarettes (Hong Jin Long; Hubei China Tobacco Industry, Hubei, China) using an animal gas-exposure apparatus (HRH-CSED-K; Beijing Huironghe Technology, Beijing, China) to maintain the cigarette concentration at 1000 mg/mm^3 for 1 h twice a day (except for weekends) for 13 months continuously. Each cigarette contained (in mg) tar (9), nicotine (0.8) and carbon monoxide (12). The protocol for exposure to cigarette smoke was to light four cigarettes, and then to add two new cigarettes every 20 min on three occasions. Control animals inhaled clean room air only in their cages. Group C was divided further into four groups of six mice: C1 (sacrificed on day 367, 7 days after tracheal instillation of MHV-68); C2 (sacrificed on day 374, 14 days after tracheal instillation of MHV-68); C3 (sacrificed on day 381, 21 days after tracheal instillation of MHV-68); C4 (sacrificed on day 388, 28 days after tracheal instillation of MHV-68). Rats from group A, B, and D were sacrificed on day 388. Then, bronchoalveolar lavage fluid (BALF) and lung specimens were collected.

2.2. Specimen processing

Mice were sacrificed by cervical dislocation after the induction of anesthesia (chloral hydrate, i.p.). Then, physiologic (0.9%) saline was injected into the right ventricles of all mice to remove blood. Lavage of the left lung was undertaken for each mouse. Right-lung tissues underwent morphometric analyses and immunohistochemical staining. Briefly, the right main bronchus was tied near the trachea and a blunt needle and 1-mL syringe inserted into the trachea. Then, the left lung was lavaged thrice with 0.6 mL of buffered saline solution, and the flow-through (final volume, 0.8–1.2 mL) was maintained on ice. BALF was centrifuged: the supernatant was stored at −80 °C for detection of cytokines (using an enzyme-linked immunosorbent assay (ELISA) kit) and the pellet was resuspended in PBS for differential cell counts as soon as possible. Next, the right lungs of all mice were inflated with 4% phosphate-buffered formalin (pH 7.2) for 3–5 min, then removed and stored in the same fixative for 48 h before embedding in paraffin. Finally, left lungs were removed and stored at −80 °C for real-time polymerase chain reaction (PCR) analyses. To avoid selection bias in morphometric analyses, right-lung tissue was maintained in the same direction when embedding in paraffin, and isotropic uniform random sections were created. Serial sections perpendicular to the right lung axis (upper–middle–basal) were cut for morphometric analyses and immunofluorescence staining.

2.3. Morphometric analyses

One set of lung paraffin sections was cut at 7.5 μm, deparaffinized, and rehydrated. Then, sections were stained with hematoxylin and eosin (H&E) or Masson's trichrome for morphometry or detection of collagen fibers, respectively. Morphologic changes in lungs were observed. Emphysematous changes were assessed by measurement of the mean linear intercept and the destructive index, as described previously (Zhang et al., 2014a), in at least eight fields for each mouse to obtain mean values. Pathologic grades of inflammation and fibrosis in each section were evaluated by three pathologists working in different hospitals who did not know the treatment program.

A five-point scale was used to ascertain the extent of fibrosis: 0 = normal lung architecture; 1 = lymphocytic infiltrates in perivascular and peribronchial areas, but no fibrosis; 2 = lymphocytic infiltrates as well as perivascular and peribronchial fibrosis; 3 = lymphocytic infiltrates and fibrotic thickening of interalveolar septa; 4 = lymphocytic infiltrates, presence of foamy macrophages, and formation of multiple fibrotic foci. Because of the patchy pathology of infected lungs, the score for 10 random fields was recorded, and the highest score found was assigned for each individual specimen (Torres-González et al., 2012).

2.4. Analyses of apoptosis in lung tissues by terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) and immunofluorescence staining

Semi-quantification of apoptotic cells in lung tissue was done using an In Situ Cell Death Detection kit (Roche, Basel, Switzerland) based on TUNEL staining according to manufacturer instructions. The number of positive signals in each section was evaluated by three pathologists working in different hospitals. Immunofluorescence images were captured using an inverted fluorescence microscope (Eclipse TE2000-U; Nikon, Tokyo, Japan) with a spot image acquisition and processing system (Nikon). By determination of the apoptosis index, the percentage of positive cells in 500 cells counted on non-overlapping scattered fields for each section was measured.

Injury to epithelial cells in the lungs is believed to be important for initiation of fibrotic processes in the lung. To ascertain the type of cells undergoing apoptosis, we undertook immunofluorescence staining using anti-pro-surfactant C antibodies as markers of type-2 lung epithelial cells (T2LECs), as described by Torres-González et al. (Torres-González et al., 2012). Also, we determined the apoptotic index of T2LECs (which is a measure of the percentage of positive T2LECs in 100 apoptotic cells).

2.5. Real-time PCR

Total RNA was extracted from 42 lung-tissue samples from mice using TRIzol® Reagent (Invitrogen, Carlsbad, CA, USA) according to manufacturer instructions. RNA samples were quantified by absorbance at 260 nm. RNA was reverse-transcribed to cDNA using a RT-PCR kit (TaKaRa Bio, Shiga, Japan) according to manufacturer instructions. cDNA was analyzed

Please cite this article in press as: Zhang, W.-G., et al., Comparative study of two models of combined pulmonary fibrosis and emphysema in mice. Acta Histochemica (2017), http://dx.doi.org/10.1016/j.acthis.2017.01.007