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Degree of endothelium injury promotes fibroelastogenesis in experimental acute lung injury

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ABSTRACT

We tested the hypothesis that at the early phase of acute lung injury (ALI) the degree of endothelium injury may predict lung parenchyma remodelling. For this purpose, two models of extrapulmonary ALI induced by *Escherichia coli* lipopolysaccharide (ALI-LPS) or cecal ligation and puncture (ALI-CLP) were developed in mice. At day 1, these models had similar degrees of lung mechanical compromise, epithelial damage, and intraperitoneal inflammation, but endothelial lesion was greater in ALI-CLP. A time course analysis revealed, at day 7: ALI-CLP had higher degrees of epithelial lesion, denudation of basement membrane, endothelial damage, elastic and collagen fibre content, neutrophils in bronchoalveolar lavage fluid (BALF), peritoneal fluid and blood, levels of interleukin-6, KC (murine analogue of IL-8), and transforming growth factor-β in BALF. Conversely, the number of lung apoptotic cells was similar in both groups. In conclusion, the intensity of fibroelastogenesis was affected by endothelium injury in addition to the maintenance of epithelial damage and intraperitoneal inflammation.

1. Introduction

Acute respiratory distress syndrome (ARDS), the most severe manifestation of acute lung injury (ALI), is typically described as a stereotyped response to lung injury with transition from alveolar-capillary damage to a fibroproliferative phase regardless of the underlying disease (Meduri, 1993; Dos Santos, 2008). Most ALI/ARDS patients survive the acute phase of lung injury and progress to either repair of the lesion or development of the syndrome (Rocco et al., 2009). Despite advances in the management of ALI/ARDS, mortality remains high (35–40%) (Phua et al., 2009). Extended pulmonary fibrosis is seen in 55% of deaths (Papazian et al., 2007), suggesting that disorganized repair may contribute to the morbidity and mortality of these patients (Fukuda et al., 1987; Martin et al., 1995).

The factors determining whether pulmonary fibrosis or restoration of the normal lung architecture will occur in ALI/ARDS remain unknown. The alveolar epithelium has been described as a critical mediator of fibroproliferation (Munger et al., 1999; Santos et al., 2006; Gropper and Wiener-Kronish, 2008; Rocco and Pelosi, 2008). Together with the epithelial barrier, the lung endothelium mediates the initial change in permeability associated with ALI/ARDS and may also be critical for the remodelling of the alveolar-capillary membrane (Orfanos et al., 2004; Maniatis et al., 2008; Maniatis and Orfanos, 2008; Lucas et al., 2009). However, the responses of lung endothelial cells are incompletely defined even though they appear to be the first cells of the lung to suffer alterations in ALI/ARDS triggered by sepsis, trauma, and other systemic conditions.

To investigate the role of endothelial cells on lung remodelling, we developed two models of extrapulmonary ALI: these models presented similar degrees of mechanical compromise, epithelial damage, and intraperitoneal inflammation, but different degrees of endothelial injury at the early phase of ALI. We hypothesized that the degree of endothelium injury would predict lung parenchyma remodelling, as well as the maintenance of epithelial damage and intraperitoneal inflammation.

2. Material and methods

This study was approved by the Ethics Committee of the Health Sciences Centre, Federal University of Rio de Janeiro. All ani-

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mals received humane care in compliance with the "Principles of Laboratory Animal Care" formulated by the National Society for Medical Research and the "Guide for the Care and Use of Laboratory Animals" prepared by the National Academy of Sciences, USA.

2.1. Animal preparation and experimental protocol

One hundred and thirty-six BALB/c mice (weighing 20–25 g) were randomly assigned to one of four groups: (1) ALI with intraperitoneal (*ip*) instillation of *E. coli* LPS (O55:B5, Sigma Chemical Co., St Louis, MO, 125 μg suspended in saline solution with total volume equal to 0.5 ml) (ALI-LPS) (Menezes et al., 2005; Santos et al., 2006; Leite-Junior et al., 2008); (2) ALI with cecal ligation and puncture (ALI-CLP) (Hubbard et al., 2005); (3) control group receiving sterile saline solution (0.9% NaCl, 0.5 ml, *ip*, C-LPS group); (4) control group submitted to sham surgery (C-CLP group). Both ALI groups were fasted for 16 h before cecal ligation and puncture-induced sepsis. Animals were anaesthetized with sevoflurane and a midline laparotomy (2 cm incision) was performed. The cecum was carefully isolated to prevent damage to blood vessels. A 3.0 cotton ligature was placed below the ileocecal valve to prevent bowel obstruction. Finally, the cecum was punctured twice with an 18-gauge needle and the animals recovered from anaesthesia (Oliveira et al., 2009). In sham surgery, the abdominal cavity was opened and the cecum was isolated without ligation and puncture. The postoperative period was similar in both cases, with animals receiving subcutaneous injections of 1 ml of warm (37 °C) normal saline with tramadol hydrochloride (20 μg/g body weight). The dose of *E. coli* LPS used in the present study has been previously described, but the number of punctures in the cecum was based on a series of pilot experiments to provide two models of ALIexp with a similar 1.5-fold increase in lung static elastance compared to control groups. Lung mechanics and histology were analyzed in 48 mice [$n = 6$/group at each time point (days 1 and 7)], while the inflammatory process in bronchoalveolar lavage fluid (BALF), blood, and peritoneal fluid was evaluated in a second group of 48 animals ($n = 6$/group at days 1 and 7). Survival studies were performed in the remaining 40 ALI mice.

2.2. Survival studies

In order to determine the survival rate, ALI-CLP ($n = 20$) and ALI-LPS ($n = 20$) animals were used. All mice had free access to water and food and were monitored during 7 days by the investigators.

2.3. Mechanical parameters

One and seven days after ALI induction, the animals were sedated (diazepam 1 mg *ip*), anaesthetized (thiopental sodium 20 mg/kg *ip*), tracheotomised, paralysed (vecuronium bromide, 0.005 mg kg^{-1} iv), and ventilated with a constant flow ventilator (Samay VR15; Universidad de la Republica, Montevideo, Uruguay) with the following parameters: frequency of 100 breaths/min, tidal volume (V_T) of 0.2 ml, and fraction of inspired oxygen of 0.21. The anterior chest wall was surgically removed and a positive end-expiratory pressure (PEEP) of 2 cm H_2O was applied. After a 10-min ventilation period, lung mechanics were computed. At the end of the experiments (approximately 30 min), lungs were prepared for histology.

Airflow and tracheal pressure (P_{tr}) were measured (Burburan et al., 2007). Lung mechanics was analyzed using the end-inflation occlusion method (Bates et al., 1988). In an open chest preparation, P_{tr} reflects transpulmonary pressure (P_L). Briefly, after end-inspiratory occlusion, there is an initial fast drop in P_L (ΔP_1) from the preocclusion value down to an inflection point (P_i), followed by a slow pressure decay (ΔP_2), until a plateau is reached. This plateau corresponds to the elastic recoil pressure of the lung (P_{el}). ΔP_1 selectively reflects the pressure used to overcome airway resistance. ΔP_2 reproduces the pressure spent by stress relaxation, or viscoelastic properties of the lung, together with a small contribution of *pendelluft*. Static lung elastance (E_{st}) was determined by dividing P_{el} by V_T. Lung mechanics measurements were performed 10 times in each animal. All data were analyzed using the ANADAT data analysis software (RHT-InfoData, Inc., Montreal, Quebec, Canada).

2.4. Lung histology

A laparotomy was done immediately after determination of lung mechanics, and heparin (1000 IU) was intravenously injected in the vena cava. The trachea was clamped at end-expiration (PEEP = 2 cm H_2O), and the abdominal aorta and vena cava were sectioned, yielding a massive haemorrhage that quickly killed the animals. The right lung was then removed, fixed in 3% buffered formaldehyde and paraffin-embedded. Four-μm-thick slices were cut and stained with haematoxylin-eosin.

Lung morphometry analysis was performed with an integrating eyepiece with a coherent system consisting of a grid with 100 points and 50 lines (known length) coupled to a conventional light microscope (Olympus BX51, Olympus Latin America-Inc., Brazil). Fraction areas of collapsed and normal lung areas were determined by the point-counting technique (Weibel, 1990; Hsia et al., 2010) across 10 random, non-coincident microscopic fields (Menezes et al., 2005; Santos et al., 2006). Polymorphonuclear (PMN) and mononuclear (MN) cells and lung tissue were evaluated at ×1000 magnification. Points falling on PMN and MN cells were counted, and divided by the total number of points falling on tissue area in each microscopic field.

Collagen (picrosirius–polarization method) and elastic fibres (Weigert's resorcin fuchsin method with oxidation) were quantified in airways and alveolar septa (Rocco et al., 2001).

2.5. Transmission electron microscopy

Three 2 mm × 2 mm × 2 mm slices were cut from three different segments of the left lung and fixed [2.5% glutaraldehyde and phosphate buffer 0.1 M (pH = 7.4)] for electron microscopy (JEOL 1010 Transmission Electron Microscope, Tokyo, Japan) analysis. For each electron microscopy image (20/animal), damage to the following structures was analyzed: (a) type I epithelial cell lesion, (b) type II epithelial cell lesion, (c) denudation of basement membrane, (d) alveolar collapse, (e) endothelial damage, (f) endothelial cell apoptosis, and (f) hyaline membrane. Pathologic findings were graded according to a 5-point semi-quantitative severity-based scoring system as: 0 = normal lung parenchyma, 1 = changes in 1–25%, 2 = changes in 26–50%, 3 = changes in 51–75%, and 4 = changes in 76–100% of examined tissue (Santos et al., 2006; Passaro et al., 2009).

2.6. Lung cell apoptosis

Terminal deoxynucleotidyl transferase biotin-dUTP nick end labelling (TUNEL) staining was performed in a blinded fashion by two pathologists to assay cellular apoptosis (Oliveira et al., 2009). Ten fields per section from the regions with cell apoptosis were examined at a magnification of ×400. A 5-point semi-quantitative severity-based scoring system was used to assess the degree of apoptosis, graded as: 0 = normal lung parenchyma; 1 = 1–25%; 2 = 26–50%; 3 = 51–75%; and 4 = 76–100% of examined tissue.