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Necrostatin-1 protects against oleic acid-induced acute respiratory distress syndrome in rats

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ABSTRACT

Necroptosis is a recently discovered necrotic cell death which is regulated by receptor interacting protein kinase 1 (RIPK1) and RIPK3 under the stimulus of death signal and can be inhibited by necrostatin-1 (Nec-1) specifically. Therefore, the aim was to investigate the role of necroptosis in a rat model of acute respiratory distress syndrome (ARDS) induced by oleic acid (OA) and assess the effect of Nec-1 on lung injury in ARDS. Our results found that RIPK1, RIPK3 and mixed lineage kinase domain-like protein (MLKL) were abundantly expressed in rat lung tissues of OA-induced ARDS. Nec-1 pretreatment improved pulmonary function and attenuated lung edema dramatically in OA-induced ARDS rats. Furthermore, Nec-1 reduced RIPK1-RIPK3 interaction and down-regulated RIPK1-RIPK3-MLKL signal pathway, and inhibited inflammatory response by reducing neutrophil infiltration and protein leakage into lung tissue in OA-induced ARDS. Collectively, our study proves the intervention of necroptosis in OA-induced ARDS. Moreover, our findings imply that Nec-1 plays an important role in the treatment of ARDS via inhibiting necroptosis and inflammation.

1. Introduction

Acute respiratory distress syndrome (ARDS) is a disease characterized by proteinaceous pulmonary edema and uncontrolled inflammation which is associated with increased permeability of the alveolar-capillary barrier [1]. Scientific clinical management has not reduced the mortality of ARDS to <30% [2]. From 1967 to now, although research on the pathophysiology and treatment of ARDS was continuously deepening, ARDS remains a major clinical problem mainly due to a lack of clearer understanding for the pathomechanism of ARDS. Necrosis and apoptosis are known as main forms of cell death in ARDS [3]. The intervention of apoptosis has been extensively studied in the pathogenesis of ARDS [4–6]. Two crucial theories link apoptosis with the pathology of ARDS including the increased alveolar epithelial cell apoptosis and the delay of neutrophil apoptosis [7]. Nevertheless, necrosis has been generally considered as an accidental and non-regulated form of cell death. Therefore, very little efforts have been made to investigate necrosis in development and progression of ARDS, in spite of its prevalence in human pathology.

Recent studies have revealed that necroptosis is a form of programed and regulated cell death [8]. Necroptosis is mediated by receptor interacting protein kinase 1 (RIPK1) and RIPK3 in the form of complex IIb through the RIP homotypic interaction motif (RHIM) [9,10]. As a substrate of RIPK3, mixed lineage kinase domain-like protein (MLKL) is an essential mediator in executing necroptosis downstream of RIPK3 [11,12]. With the help of CYLD [13], complex IIb consisting of RIPK1, RIPK3 and MLKL was finally constituted to cause necroptosis [14,15]. And necrostatin-1 (Nec-1), a specific small molecule inhibitor of necroptosis, can specifically inhibit RIPK1 kinase activity and RIPK1-RIPK3 interaction [8,10,16]. Necroptosis has been shown to take part in various disease models,

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including ischemia-reperfusion injury [17], central nervous system disease [18], and acute inflammatory reaction [19]. However, few researches about necroptosis in ARDS have been done.

Lung was the most vulnerable organ to the effects of oleic acid (OA) and the lung injury caused by OA was considered as local damage (lung tissue) rather than systemic damage [20]. Therefore, OA has been used as a common tool to study the mechanisms of ARDS. Nevertheless, it remains unclear whether RIPK1/RIPK3-mediated necroptosis occurs in OA-induced ARDS. Hence, our study was designed to investigate the role of necroptosis in a rat ARDS model induced by OA, as well as the protective effect of Nec-1 and its underlying mechanisms in ARDS rats.

2. Materials and methods

2.1. Animal model and drug administration

Male Sprague-Dawley rats, 6–8 weeks old, were purchased from Guangdong Medical Laboratory Animal Center (Foshan, Guangdong, China). Rats were randomly assigned into four groups: the group treated with dimethyl sulfoxide (DMSO) (DMSO group), the model group treated with OA (ARDS group), ARDS treated with DMSO group (ARDS + DMSO group), ARDS treated with Nec-1 group (ARDS + Nec-1 group). Each rat in experimental group was injected intravenously with pure OA at dose of 10 μl kg^{-1} 30 min after pretreatment with Nec-1 or DMSO, while control rats received an injection of an equal volume of DMSO in a warm saline solution.

For drug administration, 1 mg Nec-1 (Selleckchem, Houston, USA) was dissolved in 20 μl DMSO (Sigma, Saint Louis, USA) diluted in 980 μl warm saline solution based on previous studies [21] and our preliminary study (data not shown). Rats were pretreated with an intravenous injection of 1 mg/kg Nec-1 solution or the same volume of DMSO. Throughout the whole period, rats were kept at room temperature using a warm blanket and were sacrificed 4 h after administration. This research was carried out in accordance with the "Guideline for the Care and Use of Laboratory Animals" of China and was approved by Animal Care and Use Committee of Guangzhou Medical University.

2.2. Arterial blood gas analysis and lung wet-dry ratio

During the experimental period, the arterial blood specimens obtained from carotid artery were analyzed instantly for PaO_2, $PaCO_2$ and pH by automatic blood gas Analyzer ABL800 flex (Radiometer, Copenhagen, Denmark). The alveolar-arterial oxygen tension difference [$P(A\text{-}a)O_2$] was calculated by using the standard alveolar gas equation with an assumed respiratory quotient of 1.0. The oxygenation index was calculated by dividing PaO_2 by FiO_2.

The lung tissues was excised and weighed immediately. After washed away the blood on the lung surface, lung tissues were dried in an oven at 60 °C for 72 h and reweighed. The lung wet-dry ratio was calculated by dividing the mass of the initial specimen by the mass of the dried specimen.

2.3. Measurement and evaluation of BALF

The BALF was analyzed for protein concentrations, the assay of TNF-α, total and differential counts of leukocytes. Among them, total and differential counts of leukocytes were detected with an automatic blood cell analyzer (Sysmex, Kobe, Japan). The rest BALF was centrifuged at 1500 r/min at 4 °C for 10 min to remove the cells. The supernatant was used to determine the concentration of total protein and TNF-α. Total protein was quantified by BCA Protein Assay Kit (Beyotime, Shanghai, China). And the assay of TNF-α was done using a rat ELISA Kit (Elabscience, Wuhan, China).

2.4. Hematoxylin and eosin (H&E) and immunohistochemistry staining

Shortly after the rats were sacrificed, lung tissues were separated and soaked in 10% formalin for 24 h. Subsequently, the specimens were dehydrated, and embedded in paraffin wax, and 4 μm sections were cut and stained for H&E. To quantify the degree of lung injury, we adapted a scoring system to evaluate pulmonary architecture, leukocyte infiltration and lung edema formation from 1 to 4 (1 indicating normal and 4 representing the most severe injury) [22].

Immunohistochemistry staining was performed as our previous research [23]. The following primary diluted antibodies were used: anti-RIPK1 (Dilution, 1:50; Santa Cruz Biotechnology, Dallas, USA), anti-RIPK3 (Dilution, 1:200; Sigma-Aldrich, Saint Louis, USA), and anti-MLKL (dilution, 1:200; abcam, Cambridgeshire, UK). And we used Image-Pro Plus (Media Cybernetics, Bethesda, Maryland, USA) to evaluate the mean optical density for the quantification of immunohistochemistry staining.

2.5. Immunoprecipitation and western blot

The lung tissues were crushed into powder by liquid nitrogen and dissolved in RIPA lysis buffer (Beyotime, Shanghai, China) for 30 min on ice. Then, the lysates were centrifuged at 12000*g* for 10 min at 4 °C and supernatant were collected. Total protein concentration was assayed by BCA Protein Assay Kit (Beyotime, Shanghai, China). Immunoprecipitation was used for determining the interaction of proteins and performed as manufacturer's instructions. 1 ml lysates were added 2 μg rabbit anti-RIPK1 antibody (Santa Cruz Biotechnology, Dallas, TX, USA) and rocked slowly overnight at 4 °C. Subsequently, the lysates were mixed up with 40 μl fully resuspended Protein A + G Agarose (Beyotime, Shanghai, China) at 4 °C for 3 h with gentle rocking. The beads were washed five times with lysis buffer, and proteins were boiled with 1× loading buffer for 10 min. The specimens were evaluated by Western blot.

Western blot was performed as our previous research [23]. The primary antibodies included anti-RIPK1 (Dilution, 1:1000; Santa Cruz Biotechnology, Dallas, USA) or anti-RIPK3 (Dilution, 1:2000; Sigma-Aldrich, Saint Louis, USA) or anti-MLKL (Dilution, 1:2000; Abcam, Cambridgeshire, UK) or mouse β-actin (Dilution, 1:5000; Cell Signaling Technology, Beverly, USA).

2.6. Statistical analyses

Quantitative results were expressed as mean ± SD. The statistical analysis was determined by SPSS 16.0 (SPSS, Chicago, IL, USA). The two groups were compared using the unpaired Student's test and analysis of variance, while multiple groups were compared by one-way analysis of variance using the Tukey's multiple comparison test or the Kruskal-Wallis test followed by Dunn's multiple comparisons. P value < 0.05 was considered statistically significant.

3. Results

3.1. OA-induced ARDS was associated with necroptosis

The activation of necroptosis requires RIPK1 and RIPK3 kinase activity [24]. To see whether necroptosis occurs during ARDS, we examined RIPK1, RIPK3 and MLKL expressions in lung tissue using immunohistochemistry staining. Staining results showed that the three proteins in the lung tissue from ARDS group strongly expressed than that from Control group (Fig. 1). Also, the localization of RIPK1, RIPK3 and MLKL was mainly in cytoplasm of capillary