Original Paper

Fibrocytes Ameliorate Acute Lung Injury by Decreasing Inflammatory Cytokine and Chemokine Levels and Reducing Neutrophil Accumulation in the Lung

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Key Words

Acute lung injury (ALI) • Fibrocytes • Lipopolysaccharide (LPS) • Myeloperoxidase (MPO) • Macrophage inflammatory protein 2 (MIP-2)

Abstract

Background/Aims: Acute lung injury (ALI) remains a severe disease that threatens human life around the world. To decrease the mortality of ALI and improve ALI treatment efficacy, the development of more ALI treatments is urgently needed. Whether fibrocytes directly participate in ALI has not been studied. Therefore, a mouse model of ALI was induced with lipopolysaccharide (LPS). **Methods:** Fibrocytes were harvested from peripheral blood mononuclear cells of bleomycin mice and identified by using flow cytometry to detect the expression of molecular makers. The fibrocytes were injected for the treatment of acute lung injury mice. The curative effects were evaluated by using ELISA to determine the cytokines (including TNF- α , IL-6 and IFN- γ) concentrations in bronchoalveolar lavage fluid (BALF) supernatant. **Results:** The concentrations of cytokines such as tumor necrosis factor- α (TNF- α), interleukin-6 (IL-6), and interferon- γ (IFN- γ) were increased in mice with ALI induced with LPS. The concentrations of TNF- α , IL-6, and IFN- γ as well as their mRNA and protein expression levels were decreased by administration of fibrocytes. The effect of fibrocytes in ameliorating ALI was time dependent. LPS treatment induced an increase in myeloperoxidase (MPO) activity, whereas the fibrocyte treatment caused inhibition of MPO activity as well as expression of the neutrophil-chemoattractant chemokine macrophage inflammatory protein 2 (MIP-2). **Conclusion:** Taken together, these data suggest that fibrocytes ameliorated ALI by suppressing inflammatory cytokines and chemokines as well as by decreasing the accumulation of neutrophils in the lung.

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Introduction

Acute lung injury (ALI) remains a life-threatening disease manifesting as an acute inflammatory response of the lung and resulting in disruption of lung endothelial and epithelial barriers [1-3]. Direct injury to the lung (such as in pneumonia or aspiration) and indirect mechanisms (such as sepsis or burn) may lead to the occurrence of ALI [4]. Typical symptoms of ALI are dyspnea, tachypnea, dry cough and retrosternal discomfort [5, 6]. The present therapeutic approaches for ALI include supportive care, ventilator support, pharmacological treatments and mesenchymal stem cells (MSCs) [2, 7-9]. ALI can develop into more severe acute respiratory distress syndrome (ARDS) or multiple organ dysfunction syndrome (MODS), and the incidence and mortality of ALI remains high around the world [7, 10]. Therefore, developing novel therapies to treat ALI and improve the clinical outcomes is urgently necessary.

Lipopolysaccharide (LPS) is a key component of the cell wall of gram-negative bacteria that can induce pulmonary and systemic infection [3, 10, 11]. LPS participates in initiating the inflammatory response by binding to its receptors. LPS exposure can result in systemic inflammatory response syndrome (SIRS) and ALI [12, 13]. Therefore, LPS has been used to establish a rat ALI model in various studies. Circulating fibrocytes are derived from bone marrow and circulate within the bloodstream, and these cells are characterized by hematopoietic markers such as CD34, leukocyte markers such as CD45, and the fibroblast products α -smooth muscle actin (α -SMA) and collagen 1 (Col-1) [14]. Circulating fibrocytes migrate to inflammatory or injured sites and participate in tissue healing or repair under inflammatory conditions [15-17]. Circulating fibrocytes release vascular endothelial growth factor (VEGF), cytokines and growth factors as well as activate fibroblasts to help the repair process [15, 18]. Circulating fibrocytes can differentiate into fibroblasts, osteoblasts and adipocytes [19, 20]. Fibroblasts are important cells that participate in fibroproliferation and wound healing [21]. Previous results have shown that fibroproliferation is a stereotypical part of the normal repair process in ALI/ARDS, which is characterized by intra-alveolar accumulation of fibroblasts and collagen deposition [21-24]. Other reports have shown that excessive fibroproliferation is associated with poorer outcomes [23, 25]. Fibrocytes participate in bleomycin models of pulmonary fibrosis [26]. Fibrocytes exist in bronchoalveolar lavage fluid (BALF) and BAL blood of patients with IPF as well as the BALF of patients with ARDS, which increases mortality in ARDS patients [27]. As mentioned above, fibrocytes are closely associated with lung diseases and inflammation [16, 28]. Whether fibrocytes directly participate in ALI has not been clearly demonstrated.

To study the function of fibrocytes in ALI, LPS was used to induce ALI in mice. Then, isolated fibrocytes were administered to mice with ALI, and the efficacy of fibrocytes in treating ALI was evaluated.

Materials and Methods

Isolation of circulating fibrocytes

Fibrocytes were harvested from peripheral blood mononuclear cells (PBMC) of bleomycin mice according to previously described methods [17]. After centrifugation, the non-adherent cells were removed. Then the remained adherent cells were cultured for 10 days and the morphology of cells was observed. To confirm that the cells were indeed fibrocytes, mesenchymal markers including collagen-1, hematopoietic markers such as CD34, leukocyte markers such as CD45 and the fibroblast products α -smooth muscle actin (α -SMA) and myeloid markers were analyzed. In addition, wound healing assays were performed according to previous reports to study the therapeutic effect of isolated fibrocytes [29].

Animals

The experimental procedures were carried out according to the Guide for the Care and Use of Laboratory Animals (8th edition) [30]. Specific pathogen-free (SPF) male BALB/c mice (16-20 g) aged 6 to 8 weeks were fed under SPF conditions at room temperature (12 h dark and light cycles).

LPS-induced ALI mouse model

Forty male BALB/c mice were randomly divided into 4 groups: Control, LPS, LPS+Fb 24 h, and LPS+Fb 72 h. Based on previously published methods, ALI was induced by LPS (Sigma-Aldrich, USA) via intratracheal injection. In brief, mice were anesthetized with 30 mg/kg pentobarbital sodium and then treated with 10 µg of LPS. The mice in the Control group were administered sterile saline instead. Then, 10 minutes after LPS injection, mice were treated with Fb.

Bronchoalveolar lavage fluid (BALF) and cell counting

After 72 h, mice were sacrificed after anesthesia with pentobarbitone (50 mg/kg i.p.). BALF was collected by cannulating the upper part of the trachea by lavage 3 times with 1.0 ml of PBS (PH 7.2). The fluid recovery rate was about 90%. Lavaged samples were kept on ice, BALF was centrifuged at 4 °C. The sedimented cells were resuspended in 50 µl of PBS and stained with hematoxylin and eosin (H&E) for cytospin preparation. Total cells, neutrophils, macrophages and lymphocytes were counted in a double-blinded manner with a hemocytometer.

Determining the concentrations of TNF-α, IL-6 and IFN-γ in BALF

The BALF supernatant was collected after centrifugation (for 4 min at 4000 rpm) and stored at –80 °C for cytokine assays. Tumor necrosis factor-α (TNF-α), interleukin-6 (IL-6), and interferon-γ (IFN-γ) levels in BALF were measured by ELISA (R&D Systems, Minneapolis, MN, USA) according to the manufacturer's protocols.

Lung wet/dry weight ratio (W/D)

The severity of pulmonary edema was assessed by the wet to dry ratio (W/D). The left lower lungs were weighed and then dehydrated at 60 °C for 72 h in an oven [31].

H&E staining

The right lower lung of each mouse was fixed in 10% formalin, embedded in paraffin, cut into 5 µm sections, and stained with H&E to analyze pathological alteration of the lung tissues. The lung injury score was recorded according to previous reports [31]. In brief, a score of 0 represented no damage, 1 represented mild damage, 2 represented moderate damage, 3 represented severe damage, and 4 represented very severe histologic changes.

Reverse transcription polymerase chain reaction (RT-PCR) and Western blotting (WB)

The mRNA expression levels of TNF-α, IL-6 and IFN-γ were determined by RT-PCR. cDNA was synthesized by using PrimeScript II 1st Strand cDNA Synthesis Kit (Takara, Japan). The primer sequences used for RT-PCR were as follows: TNF-α forward primer: 5' ATGAGCACAGAAAGCATGATC 3', TNF-α reverse primer: 5' TACAGGCTTGTCACTCGAATT 3'; IL-6 forward primer: 5' GAGGATACCACTCCCAACAGACC 3', IL-6 reverse primer: 5' AAGTGCATCATCGTTGTCATACA 3'; IFN-γ forward primer: 5' ATGAACGCTACACACTGCATC 3', IFN-γ reverse primer: 5' CCATCCTTTTGCCAGTTCCTC 3'; β-actin forward primer: 5' GGCTGTATTCCCCTCCATCG 3'; β-actin reverse primer: 5' CCACTTGGTAACAATGCCATGT 3'. The PCR reactions were carried out in a 7500 Real-time PCR system (Applied Biosystems) and performed under the following thermocycler conditions: 95 °C for 20 s followed by 40 cycles of 95 °C for 3 s and 60 °C for 30 s. Raw data from all samples were collected and normalized to β-actin. The gene expression levels of TNF-α, IL-6 and IFN-γ were calculated using the relative quantification equation ($RQ=2^{-\Delta\Delta Ct}$) [32].

The protein expression of TNF-α, IL-6 and IFN-γ was evaluated by WB. Protein concentrations were determined using a BCA Protein Assay Kit (Keygen Biotech). Proteins were separated on 10% SDS-polyacrylamide gels, electroblotted onto an Immobilon-P transfer polyvinylidene fluoride membrane 20 (Millipore, USA), detected with a rabbit anti-mouse TNF-α monoclonal antibody (ab9635, 1:2000, abcam, USA), a rabbit anti-mouse IL-6 monoclonal antibody (ab6672, 1:2,000, abcam, USA), a rabbit anti-mouse IFN-γ monoclonal antibody (ab9635, 1:2000, abcam, USA), or a rabbit anti-mouse β-actin monoclonal antibody (ab8227, 1:5000, abcam, USA), and then visualized with a commercial Immobilon Western HRP Substrate (WBKLS0500, Millipore, USA) in the dark.

Myeloperoxidase (MPO) activity assay

Myeloperoxidase (MPO) activity was determined to assess the accumulation of neutrophils in the lungs. Briefly, the frozen tissue samples were thawed and suspended in 10% phosphate buffer (pH 6.0) containing 1% hexadecyltrimethylammonium bromide. The samples were sonicated on ice and centrifuged at 12000 rpm for 15 min at 4 °C. An aliquot (30 µl) was transferred into 180 µl of phosphate buffer (pH 6.0) containing 0.167 mg/mL o-dianisidine dihydrochloride and 0.0005% hydrogen peroxide (H₂O₂). The absorbance was read at 490 nm. Results are expressed as units of MPO activity per gram of lung tissue.

Statistical analysis

All statistical analyses were performed with Statistical Package for the Social Sciences (SPSS) 19.0 software. All parameters are presented as means ± SD. Statistical significance was determined by paired or unpaired Student's t-test in cases of standardized expression data. P<0.05 was considered to indicate a statistically significant difference.

Results

Identification of circulating fibrocytes in bleomycin-treated mice

To address whether circulating fibrocytes (Fbs) could treat ALI/ARDS, Fbs were first isolated from the peripheral mononuclear cells (PBMCs) of bleomycin-treated mice. The proliferation rate and the morphology of the cells is shown in Fig. 1A. Until the 5th day after isolation, long spindle-shaped cells with ellipsoid nuclei appeared, and they were considered to be Fb. After culture, most of the cells exhibited a spindle shape and adhered to the bottom of the wells.

Fbs were a distinct population of blood-borne cells and were identified by specific coexpression of several markers, including collagen-1, CD34, CD45, α-SMA and myeloid markers. On the 5th day, the percentage of CD45⁺/Col-I⁺ cells was about 9%, which represented the fibroblast precursors, while no α-SMA⁺ cells were observed at that time point (Fig. 1B & 1C). The percentage of CD45⁺/Col-I⁺ cells was increased at the 10th day, decreased beginning on the 14th day and almost zero at the 21st day. Once Fb had differentiated, the cells expressed α-SMA, a marker of myofibroblasts. The Col-I⁺/α-SMA⁺ levels increased during culture, suggesting that cultured Fb could traffic to injured tissues in response to microenvironmental stimulation.

To identify the therapeutic effects of isolated Fb, a wound-healing test was performed (Fig. 1D). The wound area narrowed by ~50% at 6 h after seeding the cells, and the wound gap was nearly covered at 24 h after seeding cells. These results suggested that the isolated Fb could differentiate into therapeutic cells *in vitro*.

Fb ameliorates pulmonary inflammation and pulmonary edema in LPS-induced acute lung injury

H&E staining was utilized to observe pathological changes in the lung tissues. As shown in Fig. 2A, the lung tissues from the LPS group demonstrated significant pathological alterations, including notable inflammatory cell infiltration, interstitial and intra-alveolar edema and patchy hemorrhaging, and some collapsed alveoli. When pre-treated with Fb, inflammatory cell infiltration was not significantly improved, but the intra-alveolar edema and patchy hemorrhaging were ameliorated remarkably. To evaluate the severity of pulmonary injury, the lung injury score and wet to dry ratio (W/D) were calculated. As shown in Fig. 2B and 2C, both the injury scores and W/D were markedly attenuated by administration of Fb. However, there was still a difference in lung injury scores and W/D between the control group and the 72 h Fb group, suggesting that Fb could only partially reverse LPS injury to the lung.

Fb decreases cell counts and inflammatory mediator levels in bronchoalveolar lavage fluid (BALF) following LPS-induced acute lung injury

To further evaluate the anti-inflammatory properties of Fb, cell counts and the levels of inflammatory mediators, including TNF-α, IL-6 and IFN-γ, in bronchoalveolar lavage

Fig. 1. Identification of circulating fibrocytes in bleomycin-treated mice. A. The cell proliferation rate and cell morphology. B and C. The percentage of CD45⁺/Col-I⁺ and Col-I⁺/α-SMA⁺ cells among fibroblasts (6 mice/group). D. Wound healing assay of isolated fibroblasts. The data are presented as the mean ± SEM. Statistical significance was determined by paired or unpaired Student's t-test. * represented p<0.05 significant difference compared to Day 5; ** represented p<0.01 significant difference compared to Day 5.

Fig. 2. Fb ameliorate pulmonary inflammation and pulmonary edema in LPS-induced ALI. A. Pathological changes in the lung tissues observed by H&E staining. B and C. The lung injury score and lung weight/dry weight ratio (W/D) (6 mice/group). The data are presented as the mean $\pm$ SEM. Statistical significance was determined by paired or unpaired Student's t-test. * represented $p < 0.05$ significant difference compared to Control group; # represented $p < 0.05$ significant difference compared to LPS group.

Fig. 3. Fb decrease cell counts and the levels of inflammatory mediators in bronchoalveolar lavage fluid (BALF) after LPS-induced acute lung injury (6 mice/group). A. The numbers of total cells, neutrophils and macrophages in BALF. B. The levels of TNF- α , IL-6, and IFN- γ in BALF. C. The levels of TNF- α , IL-6, and IFN- γ in serum. The data are presented as the mean $\pm$ SEM. Statistical significance was determined by paired or unpaired Student's t-test. * represented $p < 0.05$ significant difference compared to Control group; # represented $p < 0.05$ significant difference compared to LPS group.

Fig. 4. Effect of Fb pre-treatment on cytokine profiles. A. The mRNA expression levels of TNF- α , IL-6, and IFN- γ in serum and lung extracts. B. The protein expression levels of TNF- α , IL-6, and IFN- γ in serum and lung extracts. The data are presented as the mean $\pm$ SEM.

fluid (BALF) were measured. As shown in Fig. 3, the numbers of total cells, neutrophils and macrophages in BALF were significantly increased after LPS stimulation. Fb largely reduced the number of total cells, neutrophils, macrophages in BALF 72 h after treatment with Fb (Fig. 3A). However, there were significant differences in the total cells, neutrophils, and macrophages between the control group and the LPS+Fb 24 h intervention group, which suggested that Fb could markedly reduce the inflammatory response caused by LPS but could not completely prevent the inflammation. Differences in lymphocyte levels in BALF were not found among all groups. In addition, the levels of TNF- α , IL-6, and IFN- γ in BALF were consistent with the number of neutrophils (Fig. 3B).

To explore the effect of Fb on serum cytokine profiles, serum samples were collected from LPS or Fb pre-treated mice after intervention. All cytokine levels in serum markedly increased after LPS injection and then decreased after administration of Fb (Fig. 3C). However, the cytokine levels at 24 h and 72 h were still higher than those of the control group ($p < 0.05$). The results were similar to the cytokine levels in BALF.

Effect of Fb pre-treatment on cytokine profiles

Fb pre-treatment significantly suppressed neutrophil accumulation in the lung caused by LPS-induced ALI. To demonstrate the effect of Fb pre-treatment on serum chemokine profiles, the profile of cytokine expression in serum and lung extraction was determined by qPCR and Western blot, and we examined the difference between the control and pre-treatment groups. As shown in Fig. 4, the expression levels of TNF- α , IL-6, and IFN- γ were elevated in serum and the lung after stimulation with LPS but were suppressed by administration of Fb. The expression levels of these factors at 72 h were lower than those at 24 h but still higher than those in the control group.

Fig. 5. Fb inhibit myeloperoxidase (MPO) activity and chemokine levels following LPS-induced lung injury (6 mice/group). A. Myeloperoxidase (MPO) activity after administration of Fb. B. The levels of MIG (CXCL9), MIP-2 (CXCL2) and RANTES (CCL5) were affected by treatment with Fb. The data are presented as the mean $\pm$ SEM. Statistical significance was determined by paired or unpaired Student's t-test. * represented $p < 0.05$ significant difference compared to Control group; # represented $p < 0.05$ significant difference compared to LPS group.

Fb inhibit myeloperoxidase (MPO) activity and chemokine expression following LPS-induced lung injury

Myeloperoxidase (MPO) activity was used to assess the activation and accumulation of neutrophils in lung tissues. After LPS injection, MPO activity was significantly enhanced, and MPO activity was inhibited by treatment with Fb (Fig. 5A). As shown in Fig. 5B, the levels of MIG (CXCL9), MIP-2 (CXCL2) and RANTES (CCL5) in serum were significantly suppressed by treatment with Fb. Notably, the expression of the neutrophil-chemoattractant chemokine MIP-2 was suppressed. Taken together, our present data suggested that Fb pre-treatment may suppress the expression of inflammatory cytokines and chemokines, resulting in decreased accumulation of neutrophils in the lung.

Discussion

ALI remains a severe disease that threatens human life around the world and manifests as abnormal gas exchange or chest pain [2]. Pro-inflammatory cytokines such as TNF- α , IL-6, and IFN- γ , which have strong pro-inflammatory activities, are often used as biomarkers of ALI [33-35]. The levels of TNF- α , IL-6, and IFN- γ are increased in the early phase of ALI [33-35]. The activity of myeloperoxidase (MPO), a lung injury marker, can be used to evaluate the accumulation of neutrophils in the lungs [36]. Serum chemokines, such as MIG (CXCL9), MIP-2 (CXCL2) and RANTES (CCL5), are related to lung injury [37-40]. LPS is generally used to induce ALI in mice. To decrease the mortality of ALI and improve ALI treatment, the development of novel treatments for ALI is urgently needed. Fibrocytes are a type of cell that is related to inflammation and wound healing [15, 16]. Fibrocytes are closely associated with lung diseases and inflammation [14, 16, 28, 41, 42]. Whether fibrocytes directly participate in ALI has not been studied previously.

Herein, a mouse ALI model was induced with LPS. The concentrations of cytokines such as TNF- α , IL-6, and IFN- γ were increased in the LPS group compared with the control group and the LPS+Fb group at the same time points, which was consistent with previous

results [34]. Isolated fibrocytes were administered to mice with ALI, and the concentrations of TNF- α , IL-6, and IFN- γ were determined. The results showed that the concentrations of TNF- α , IL-6, and IFN- γ , as well as their mRNA and protein expression levels, were decreased by fibrocytes. The effect of fibrocyte-mediated amelioration of ALI is time dependent. LPS treatment induced the enhancement of MPO activity, whereas fibrocyte treatment caused the inhibition of MPO activity as well as a reduction in the levels of other serum chemokines such as MIG (CXCL9), MIP-2 (CXCL2) and RANTES (CCL5). These results suggested that fibrocytes could ameliorate the inflammation associated with ALI and repair ALI-mediated injury [43]. Furthermore, the expression of the neutrophil-chemoattractant chemokine MIP-2 was suppressed by administration of Fb. Taken together, these data suggested that fibrocytes ameliorated ALI by suppressing inflammatory cytokines as well as chemokines and decreasing the accumulation of neutrophils in the lung.

Fibrocytes can differentiate into fibroblasts, which are the key cells that participate in fibroproliferation and wound healing [19, 23]. Fibroproliferation is an important process in ALI, which is considered to be the injury repair process for ALI.¹⁵ However, many reports have shown that excessive fibroproliferation induces fibrosis of the lung and causes the death of patients with ALI [21, 24]. In addition, another report showed that the alveolar fibrocyte level can act as an independent predictor of poor outcomes in patients with ALI [27]. Therefore, the function of fibrocytes in ALI needs to be further investigated in future studies.

Conclusion

Taken together, fibrocytes decreased the levels of pro-inflammatory cytokines such as TNF- α , IL-6, and IFN- γ . Furthermore, fibrocyte treatment caused the inhibition of the MPO activity, a reduction in the levels of other serum chemokines such as MIG (CXCL9), MIP-2 (CXCL2) and RANTES (CCL5) as well as chemokines, and a decrease in the accumulation of neutrophils in the lung. Therefore, we conclude that fibrocytes indeed ameliorate ALI-mediated inflammation and can repair ALI-induced injury.

Disclosure Statement

The authors declare that there is no conflict of interest associated with this work.

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