Original Article

Roles of MiR-21 in lung tissues of lipopolysaccharide induced ALI/ARDS rats and its correlation with TNF-α

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Received March 29, 2016; Accepted May 10, 2016; Epub November 15, 2016; Published November 30, 2016

Abstract: Objective: To explore the roles of miR-21 in lung tissues of lipopolysaccharide (LPS) induced ALI/ARDS rats with experiments in vivo and in vitro, and to identify the effect of miR-21 on TNF-α expression in acute lung injury. Methods: Mice lung macrophage cell line MH-S was cultured in vitro, after the stimulation with LPS for 0 h, 3 h, 6 h and 12 h, the expression of miR-21 and TNF-α in MH-S cell line was determined by real-time quantitative PCR; miR-21 mimic was used to transfected MH-S cell line, and real-time quantitative PCR was used to detect the expression change of TNF-α at different time points; ALI/ARDS mouse model was constructed and appraised, then ELISA was used to detect TNF-α protein expression in bronchoalveolar lavage fluid; real-time quantitative PCR was used to detect the expression changes of miR-21 and TNF-α mRNA in lung tissues. Results: The mRNA expression of TNF-α in LPS-induced MH-S cells reached its highest level at 3 h (66.51 ± 23.45 times), and then gradually reduced after 6 h; and the expression of miR-21 began to rise at 6 h after stimulation (4.89 ± 0.75 times). Over-expression of miR-21 mimics down-regulated the mRNA expression of TNF-α to 0.42 ± 0.05 times. The ALI/ARDS mouse model was successfully constructed, TNF-α concentration in bronchoalveolar lavage fluid of ALI/ARDS group (490.54 ± 46.8 pg/ml) was significantly higher than that of the control group (47.62 ± 14.22 pg/ml) (P < 0.01); Compared with control group, the expression of TNF-α mRNA in ALI/ARDS group raised 3.15 ± 0.95 times (P < 0.05) and 1.85 ± 0.18 times (P < 0.05), respectively. Conclusions: In the process of ALI/ARDS, up-regulation of miR-21 in alveolar macrophages could reduce the release of inflammatory cytokine TNF-α to alleviate lung inflammation.

Keywords: microRNA-21, TNF-α, inflammatory markers, disease severity, sever pneumonia

Introduction

Acute Lung Injury (ALI)/Acute Respiratory Distress Syndrome (ARDS) is a clinical complication characterized with acute, progressive and intractable hypoxemia, it is currently one of the main causes of death in critically ill patients [1] with a mortality rate as high as 30% to 50% [2]. Currently, the main treatments for ALI/ARDS are lung protective ventilation, extracorporeal membrane oxygenation and high volume hemofiltration etc. [3]. However, the clinical efficacy is not satisfied. Thus, the exploration of the pathogenesis of ALI/ARDS has been one of the hot spots in respiratory department [4].

In recent years, the roles of microRNA-21 in innate immune response regulation have drawn more and more attention. MiR-21, by target silencing the signal protein in signaling pathway, can inhibit the release of inflammatory cytokines and thus inhibit the excessive activation of alveolar macrophages and lower down alveolar inflammation [5, 6]. In this experiment, by constructing ALI/ARDS mouse model and cell experiments, we first observed the expression of miR-21 in damaged lung tissues and its influence on the expression of TNF-α, and then explored its regulatory role and mechanism in ALI/ARDS mouse model to provide new treatment ideas for clinical treatment of ALI/ARDS.

Materials and methods

Experimental animals

6-week-old male C57BL/6J mice with an average body weight of 20.12 ± 5.32 g were pro-
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vided by the experimental animal center of Zhengzhou University.

Main reagents and instruments

Mouse alveolar macrophage cell line MH-S (purchased from ATCC company, USA), LPS (purchased from Sigma-Aldrich Inc., USA); TNF-α kit (purchased from Shanghai Ming Rui biological Co. Ltd.); TaqMan®MicroRNA Reverse Transcription Kit (purchased from ABI Inc., USA); gradient PCR instrument (purchased from TaKaRa Inc., USA); real-time quantitative PCR instrument (purchased from Agilent Technologies Inc., USA); horizontal electrophoresis tank (Bio-Rad, Inc., USA); enzyme standard instrument (purchased from ThermoFisher Inc., USA).

Methods

Expression of miR-21 and TNF-α in LPS induced MH-S cells: MH-S cells at logarithmic phase were selected to prepare single-cell suspension; the cells were inoculated into six-well plate with a concentration of 2×10^6 cells in each hole and cultured with PRMI1640 culture medium containing 10% fetal bovine serum. LPS, miR-21 mimics and miR-21 mimics control at final concentration of 1 μg/ml were added into the culture medium respectively, and the cells were divided into three groups: LPS stimulation group, miR-21 mimics transfection group and miR-21 mimics control transfection group. At the different time points of 0 h, 3 h, 6 h and 12 h, the cells were centrifuged and collected, and the expression changes of miR-21 and TNF-α were determined by RT-qPCR.

RT-qPCR experiment: Total RNA was extracted with Trizol reagent and used to synthesize cDNA under the action of reverse transcriptase. β-actin was used as the internal control. β-actin forward primer: 5’-CTGTCCCTGTATGCCTCTG-3’, reverse primer: 5’-ATGTCACGCAGATTTCC-3’. TNF-α forward primer: 5’-AGCCGATGGTATTGTA-3’, reverse primer: 5’-ACTTGGGCAGATTGA-3’. 20-μL reaction system: 2 ul cDNA, 0.8 ul for each forward and reverse primer, SYBR Premix Ex TaqTM II (2×) 10 ul, ROX Reference Dye (50×) 0.4 ul, dH₂O 6 ul. After centrifugation, Applied Biosystems 7500 quantitative PCR instrument was inserted for amplification. Reaction conditions: pre-degeneration at 95°C for 30 s, degeneration at 95°C for 5 s, annealing at 57°C for 20 s, and extent at 72°C for 27 s, repeat for 40 cycles. β-actin was used as a reference gene, and 2^-ΔΔCt method was used to calculate the relative expression of mRNA in each group. After reverse transcription of miR-21, the PCR amplification reaction system was established as follow: TaqMan2xUniversal PCR Master Mix 10 ul, TaqMan MicroRNA Assay 1 ul, cDNA 1.4 ul, RNase-Free Water 7.6 ul. Reaction conditions: 95°C for 10 min, 95°C for 15 sec and 60°C for 1 min. U6snRNA was selected as the internal control, and 2^-ΔΔCt method was used to calculate the relative expression of miR-21 in each group.

The establishment of ALI/ARDS mouse model: 20 male mice were randomly divided into ALI/ARDS group and control group with 10 rats in each group. The mice were fasted but drank ad libitum on the first night before the model was constructed. 3% amobarbital (100 mg/kg) was intraperitoneally injected for anesthesia. Limbs and head of mice were fixed, and a longitudinal incision of about 0.5 cm along the neck was created to expose the trachea. 7.5 mg/kg LPS solution was injected into the trachea of ALI/ARDS mice by using the 1 ml syringe. The control group was injected with equal amount of sterile normal saline. After the injection, the incision was sutured and the mice were put into culturing chamber. 6 h later, the abdominal cavities of mice were opened following anesthesia procedures mentioned above, about 0.5 ml arterial blood was extracted to undergo arterial blood gas analysis, then, the animals were sacrificed. Bronchoalveolar lavage was performed after ligation of the right lung; the left lung tissues were cut off and placed in liquid nitrogen immediately to extract RNA, or fixed with 4% paraformaldehyde and subjected to HE staining.

Determination of TNF-α in bronchoalveolar lavage fluid: After sampling of lung tissues, 5 ml of normal saline was injected into the lungs and preserved for 2-3 min before extracting, repeat the cycle for 5 times. Lavage fluid was preserved at -20°C. TNF-α concentration in lavage fluid was determined by ELISA.

Statistical analysis

SPSS17.0 software was used for statistical analysis; measurement data were expressed as mean ± standard deviations, comparison between groups was examined by t-test or
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ANOVA; count data were expressed as percentage, and the comparison between groups was examined by chi-square test. \( P < 0.05 \) was considered to be statistically significant.

Results

Changes in mRNA expression of TNF-α in LPS-induced MH-S cells

After LPS stimulation, the TNF-α expression levels in MH-S cells at 0 h, 3 h and 12 h were 66.51 ± 23.45 times, 28.31 ± 7.93 times and 10.82 ± 4.65 times of the level at 0 h, respectively. As we can see, LPS could induce the expression of miR-21 in MH-S cells, and the expression gradually increased from 6 h after stimulation, see Figure 1.

Changes in the expression of miR-21 in LPS-induced MH-S cells

miR-21 mimics and miR-21 mimics control were used to transfect MH-S cells, after the transfection, 1 μg/ml LPS was used to stimulate MH-S cells for 6 h, cells were collected to detect the expression of TNF-α mRNA. Compared with the control group, the mRNA expression of TNF-α in miR-21 mimics group was down-regulated to 0.42 ± 0.05 times (Figure 2). Visibly, over-expression of miR-21 can reduce the expression of TNF-α in LPS-induced MH-S cells.

ALI/ARDS model of Mice

Mice of ALI/ARDS group were generally in poor condition with less activity, cyanosis on limbs and mucous membrane around the nose and mouth, short breath and wheezing; mice of control group were under normal condition with regular activity and breath and rosy on limbs and mucosa around the nose and mouth. Specimens from the lung tissues: the lung volume of the mice in ALI/ARDS group was significantly increased, the color of the lung tissue was blood red with a large amount of scattered bleeding points, pulmonary edge was blunt with edema; the mice of control group had pink, normal volume lung without bleeding point or significant edema.
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6 h after tracheal instillation of LPS, abdominal aortic blood gas analysis showed that the PaO₂ and oxygenation index (PaO₂/FiO₂) of the mice in ALI/ARDS group was significantly lower than that of control group (P < 0.01), and the oxygenation index in ALI/ARDS group was less than 300 mmHg, which meets the clinical diagnostic criteria of ALI/ARDS; however, the difference in PaCO₂ between the two groups was not statistically significant (P > 0.05). See Table 1.

HE staining of lung tissue

Results from HE staining of lung tissues: In ALI/ARDS group, the lung tissues showed significant pulmonary hemorrhage, a large number of neutrophil infiltration, alveolar septal thickening and thrombosis in the pulmonary capillaries and alveolar dilation; however, in control group, the lung tissues were in normal structure without obvious pathological damage. See Figure 4.

Expression of TNF-α and miR-21 in mice lung tissue

Expression of TNF-α and miR-21 in mice lung tissue was detected using qRT-PCR method. The results showed that compared with the control group, the mRNA expression of TNF-α in ALI/ARDS group was up-regulated to 3.15 ± 0.95 times (P < 0.05), and the expression of miR-21 was up-regulated to 1.85 ± 0.18 times (P < 0.05), See Figure 6.

Discussion

There are many factors that lead to ALI/ARDS, mainly including intra-pulmonary and extra-pulmonary factors [7]. Intrapulmonary factors include pulmonary contusion, pulmonary edema, pneumonia and so on; extra-pulmonary factors include sepsis, acute pancreatitis, diabetes and so on [8, 9]. Among them, sepsis and pneumonia are the main causes of ALI/ARDS. Lipopolysaccharide (LPS), the main pathogenic component of gram negative bacteria, can combine with monocyte and Toll like receptor 4 (TLR4) on macrophage membrane and results in the release of a large number of inflammatory cytokines through a series of signal transduction, leading to sepsis and pneumonia [10]. Directly titrating of LPS in respiratory tract can cause lung tissue necrosis and edema, and activate a large number inflammatory cells in lung tissue to induce lung inflammation; LPS induced inflammation is suitable for the in vivo

Table 1. Blood gas analysis of ALI/ARDS group and control group (mean ± SD, n=10 for each group)

<table>
<thead>
<tr>
<th>Group</th>
<th>PO₂ (mmHg)</th>
<th>PaO₂/FiO₂ (mmHg)</th>
<th>PCO₂ (mmHg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>ALI/ARDS group</td>
<td>57.34 ± 1.23*</td>
<td>246.35 ± 13.24*</td>
<td>45.34 ± 2.34</td>
</tr>
<tr>
<td>Control group</td>
<td>81.34 ± 2.35</td>
<td>456.13 ± 12.34</td>
<td>44.24 ± 3.45</td>
</tr>
</tbody>
</table>

*P < 0.01, compared with the control group.
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The study of ALI/ARDS model [11, 12]. This experiment used intra-tracheal titrating of LPS solution to construct the ALI/ARDS model. In the process of ALI/ARDS, the main cause of lung tissue damage was a large number of inflammatory cytokines acting on lung tissues. Among them, TNF-α, secreted by activated alveolar macrophages and neutrophils, is one of the important inflammatory cytokines [13]. TNF-α can directly destroy the alveolar wall, in the meanwhile, it also can stimulate lung tissues to release proteolytic enzymes and active oxygen etc. to indirectly injure the lung tissue cells [14, 15].

In recent years, the role of microRNAs (miRNAs) in the regulation of inflammatory response has drawn more and more attention and been widely researched [16, 17]. miRNAs is a type of endogenous non-coding RNA [18, 19] with about 22 nucleotides in length. In mammals, miRNAs is involved in a variety of biological processes, such as cell differentiation, development of cancer, and so on. More importantly, studies have confirmed that miRNA is closely related to the inflammatory response [20-22].

The research results showed that ALI/ARDS mouse model was successfully established 6 hours after intra-tracheal titration of LPS. The mice were generally in poor condition with less activity, cyanosis on limbs and peripheral membrane of mouth and nose, and short breath. The anatomical study of thoracic cavity showed the lung was in bloody red with scattered bleeding points and obvious edema. The results of HE staining showed that the lung tissues had significant pulmonary hemorrhage, a large number of neutrophilic granulocyte infiltrate, alveolar septa thickening, alveolar dilation, and thrombosis in pulmonary capillary. Blood gas analysis showed that oxygen partial pressure was reduced, and the oxygenation index was decreased significantly. On the level of molecular biology, the TNF-α mRNA expression in LPS-induced MH-S cells reached its peak at 3 h (66.51 ± 23.45 times), and gradually reduced 6 h later; the expression of miR-21 started to increase at 6 h (4.89 ± 0.75 times), thus, we can see that miR-21 expression was negatively correlate with mRNA expression of TNF-α. Studies have shown that miR-21 can reduce the release of monocyte inflammatory cytokines, and our study indicated that miR-21 might be involved in the regulation of inflammatory response in LPS-induced MH-S cell model [23]. In this study, by transfecting cells, we proved that over-expression of miR-21 down-regulated the mRNA expression of TNF-α to 0.42 ± 0.05 times, indicating miR-21 restrains the mechanism of releasing TNF-α in LPS-induced MH-S cells. The results of animal experiments showed that TNF-α concentration (490.54 ± 46.8 pg/ml) in the lavage fluid of ALI/ARDS group was significantly higher than that of control group (47.62 ± 14.22 pg/ml); compared with the control group, the mRNA expression of TNF-α in ALI/ARDS group was up-regulated to 3.15 ± 0.95 times (P < 0.05), and the expression of miR-21 was up-regulated to 1.85 ± 0.18 times (P < 0.05), indicating that miR-21 involved in the pulmonary inflammatory response, and had correlation with the mRNA expression of TNF-α.

Figure 6. Comparison of the expressions of TNF-α mRNA and miR-21 in lung tissue of two groups, *P < 0.05.
To sum up, in the process of ALI/ARDS, up-regulating the expression of miR-21 in lung tissues can reduce the release of lung inflammatory factor TNF-α, and further reduce the lung inflammatory reaction. Therefore, miR-21, as a new therapeutic target of ALI/ARDS, has high clinical value that worth further study.

Disclosure of conflict of interest
None.

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