Mesenchymal Stem Cells Alleviate LPS-Induced Acute Lung Injury in Mice by MiR-142a-5p-Controlled Pulmonary Endothelial Cell Autophagy

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Key Words
Mesenchymal stem cells (MSCs) • Acute lung injury (ALI) • Beclin-1 • MiR-142a-5p • Lipopolysaccharides (LPS)

Abstract
Background/Aims: Damages of pulmonary endothelial cells (PECs) represent a critical pathological process during acute lung injury (ALI), and precede pulmonary epithelial cell injury, and long-term lung dysfunction. Transplantation of mesenchymal stem cells (MSCs) has proven therapeutic effects on ALI, whereas the underlying mechanisms remain ill-defined.

Method: We transplanted MSCs in mice and then induced ALI using Lipopolysaccharides (LPS). We analyzed the changes in permeability index and lung histology. Mouse PECs were isolated by flow cytometry based on CD31 expression and then analyzed for autophagy-associated factors LC3 and Beclin-1 by Western blot. Beclin-1 mRNA was determined by RT-qPCR. In vitro, we performed bioinformatics analyses to identify the MSCs-regulated miRNAs that target Beclin-1, and confirmed that the binding was functional by 3'-UTR luciferase reporter assay.

Results: We found that MSCs transplantation significantly reduced the severity of LPS-induced ALI in mice. MSCs increased autophagy of PECs to promote PEC survival. MSCs increased Beclin-1 protein but not mRNA. MiR-142a-5p was found to target the 3'-UTR of Beclin-1 mRNA to inhibit its protein translation in PECs. MSCs reduced the levels of miR-142a-5p in PECs from LPS-treated mice. Conclusion: MSCs may alleviate LPS-ALI through downregulation of miR-142a-5p, which allows PECs to increase Beclin-1-mediated cell autophagy.

Z. Zhou and Z. You contributed equally to this work.
Introduction

Acute lung injury (ALI) is a common disease that may lead to development of highly lethal acute respiratory distress syndrome (ARDS) in humans [1-4]. The cellular pathology of ALI includes breakdown of alveolar-capillary membrane integrity, excessive neutrophil migration and production and secretion of pro-inflammatory cytokines, e.g. CXCL1, CXCL2, interleukin (IL)-6 and tumor necrosis factor (TNF)-α [5-7]. Injuries occur to both pulmonary endothelial cells (PECs) and alveolar epithelial cells after ALI, in which the recovery of the disease could also result in loss of parts of the lung function [1-4].

Mesenchymal stem cells (MSCs) have been initially identified in the bone marrow, and are the stem-like cells that expand in culture and may differentiate into osteoblasts, chondrocytes, and adipocytes [8-14]. Clinical data provide evidence to demonstrate a therapeutic effect of transplantation of MSCs in some diseases, including ALI, in which MSCs promote tissue repair through various mechanisms [9-14]. When MSCs are intravenously delivered, they are mainly localized in the lungs, as a privilege for them to be used for treating lung diseases [15]. However, the molecular mechanisms underlying the therapeutic effects of MSCs in lung diseases, e.g. ALI, are largely undetermined.

Autophagy is a catabolic pathway to degrade and recycle cellular compartments for cell survival at nutrient deprivation on physiological cellular metabolism, sometimes as a survival method for cancer cells [16-18]. During autophagy, autophagosomes engulf cytoplasmic components, resulting in conjugation of a cytosolic form of microtubule-associated protein 1A/1B-light chain 3 (LC3-I) to form LC3-phosphatidylethanolamine conjugate (LC3-II). Thus, the ratio of LC3-II to LC3-I represents the autophagic activity [16-18]. Autophagy-associated protein 6 (ATG6, or Beclin-1) is the most important autophagy regulator, and upregulation of Beclin-1 generally represent increases in autophagic activities [19].

MicroRNA (miRNA) is a class of non-coding small RNA that specifically recognizes the 3′-untranslated region (3′-UTR) of the mRNA of some genes through nucleotide pairing [20, 21]. MiRNAs play a critical role in various physiological and pathological events. Among all miRNAs, miR-142a-5p is a newly recognized one and has just been associated with peripheral blood mononuclear cells that affect from chronic antibody-mediated rejection in renal transplant patients [22]. Its role in MSCs/ALI, however, has not been acknowledged.

Here, we found that MSCs transplantation significantly reduced the severity of LPS-induced ALI in mice. MSCs increased autophagy of PECs to promote PEC survival. MSCs increased Beclin-1 protein but not mRNA. MiR-142a-5p was found to target the 3′-UTR of Beclin-1 mRNA to inhibit its protein translation in PECs. MSCs reduced the levels of miR-142a-5p in PECs from LPS-treated mice.

Materials and Methods

MSCs isolation, culturing and differentiation

The MSCs were isolated and grown in culture. Briefly, bone marrow from C57BL/6 mice were dispersed in DMEM and then centrifuged at 900 g for 5 min. The pellets were re-suspended and plated at 10⁵ cells/cm² in DMEM containing 10% heat-inactivated fetal bovine serum (FBS; Sigma-Aldrich, St Louis, MO, USA), 100 U/ml penicillin, and 100 μg/ml streptomycin (Invitrogen, Carlsbad, CA, USA) in a humidified atmosphere of 5% CO₂ at 37°C. A clone was selected after subjected to chondrogenetic, osteogenic, and adipogenic differentiation assays to confirm a MSC phenotype. For chondrogenetic induction, 2.5 × 10⁵ MSCs were induced with 5 ml chondrogenetic induction medium containing 10 μg TGFβ1 (R&D System, Los Angeles, CA, USA), 50 μg insulin-like growth factor 1 (R&D System), and 2 mg/ml dexamethasone (DMSO, Sigma-Aldrich) followed by centrifugation at 500g for 5 min. The cell pellets were maintained in the chondrogenetic induction medium for 14 days and subjected to Alcian blue staining. For osteogenic induction, cells were digested and seeded onto a 24-well plate at a density of 104 cells/well, and then maintained in osteogenic induction medium containing 10 nM Vitamin D₃ (Sigma-Aldrich) and 10 mM β-phosphoglycerol and 0.1 μM DMSO for 14 days and were subjected to Von kossa staining. For adipogenic induction, cells were digested and
seeded onto a 24-well plate at a density of $10^4$ cells/well, and then maintained in the adipogenic induction medium containing 0.5 mM 3-isobutyl-1-methylxanthine (IBMX, Sigma-Aldrich), 200 µM indomethacin, 10 µM insulin and 1 µM DMSO for 14 days and subjected to Oil red O staining.

**Cell lines**

Human lung epithelial cell line HULEC-5a (HULEC) [23] was purchased from ATCC (American Type Culture Collection, Manassas, VA, USA). HULEC-5a is an immortalized human microvascular endothelial cell line, and was maintained in MCDB131 media (without L-Glutamine, ATCC) supplemented with 10 ng/ml Epidermal Growth Factor (EGF, Sigma-Aldrich), 1 µg/ml Hydrocortisone (Sigma-Aldrich), 10 mmol/l Glutamine (Invitrogen, Carlsbad, CA, USA) and 10% fetal bovine serum (FBS, Invitrogen). HULEC cells were incubated in a humidified chamber with 5% CO$_2$ at 37°C.

**Animal manipulations**

All mouse experiment protocols were approved by the Animal Research and Care Committee at the Sixth People’s Hospital of Zhengzhou. All experiments were performed in accordance with the guidelines from the Animal Research and Care Committee at the Sixth People’s Hospital of Zhengzhou. Specific pathogen free (SPF) C57/BL6 mice (female, aged 10 weeks, weight 20 – 24 g) were supplied by Laboratory Animal Center of Shanghai Academy of Sciences, Chinese Academy of Sciences, China. For MSCs transplantation, $10^6$ cells were i.v. injected. One week later, 50 µg LPS from Escherichia coli (serotype O111:B4; Sigma-Aldrich) in 40 µl PBS was given intratracheally to the mice with/without MSCs transplantation (LPS; LPS+MSCs). A group of mice that did not receive either LPS (but saline as control) or MSCs was also used as a control (Saline).

**Permeability index**

For retrieval of bronchoalveolar lavage fluids (BALF), airways were flushed with 0.8 ml PBS. If not otherwise noted, the permeability index was determined and BAL fluids were collected 6 h after lung injury induction. Permeability index is a quantitative marker for vascular leakage. Briefly, BSA was labeled with 125I by the chloramine T method. A trace amount of 125I-BSA (specific activity 5 µCi/µg) was added to unlabeled BSA (5 mg/ml in PBS), and 200 µl of this solution was injected intravenously. Four hours later, mice were euthanized with i.p. ketamine and blood was collected from the inferior vena cava. The thorax was opened, left atrium incised, and the lung was perfused in situ with PBS via the pulmonary artery. The flushed lungs were removed and permeability index (indicating the extent of pulmonary leakage) was determined by using a gamma counter and expressed as the ratio of counts per min (cpm) in the whole lung versus radioactivity in 100 µl of blood.

**Cell transfections**

MiR-142a-5p mimics (miR-142a-5p), miR-142a-5p antisense oligonucleotides (as-miR-142a-5p) and control null sequence (null) were obtained from Origene (Beijing, China). These plasmids were used to modify miR-142a-5p levels, and transfected into HULEC cells at a concentration of 50 nmol/l, at a MOI of 100 for 12 hours, using Lipofectamine-2000 (Invitrogen) according to the manufacturer’s instructions.

**RNA extraction, reverse transcription and quantitative RT-PCR (RT-qPCR)**

Total RNA was extracted from sorted or cultured cells with miRNeasy mini kit (Qiagen, Hilden, Germany). Complementary DNA (cDNA) was randomly primed from 2µg of total RNA using the Omniscript reverse transcription kit (Qiagen). Quantitative PCR (RT-qPCR) was performed in duplicates with QuantiTect SYBR Green PCR Kit (Qiagen). All primers were purchased from Qiagen. Data were collected and analyzed with the Rotorgene software accompanying the PCR machine, using 2$^{-\Delta\Delta C_{t}}$ method for quantification of the relative mRNA expression levels. Values of genes were first normalized against α-tubulin, and then compared to controls.

**Western blot**

The protein was extracted using RIPA lysis buffer (1% NP40, 0.1% Sodium dodecyl sulfate (SDS), 100 µg/ml phenylmethylsulfonyl fluoride, 0.5% sodium deoxycholate, in PBS) on ice. The supernatants were collected after centrifugation at 12,000g at 4°C for 20 min. Protein concentration was determined
using a BCA protein assay kit (Bio-rad, China), and whole lysates were mixed with 4 × SDS loading buffer (125 mmol/l Tris-HCl, 4% SDS, 20% glycerol, 100 mmol/l Dithiothreitol (DTT), and 0.2% bromophenol blue) at a ratio of 1:3. Samples were heated at 100°C for 5 min and were separated on SDS-polyacrylamide gels. The separated proteins were then transferred to a PVDF membrane. The membrane blots were first probed with a primary antibody. After incubation with horseradish peroxidase-conjugated second antibody, autoradiograms were prepared using the enhanced chemiluminescent system to visualize the protein antigen. The signals were recorded using X-ray film. Primary antibodies were anti-LC3, anti-Beclin-1 and anti-α-tubulin (all purchased from Cell Signaling, St Louis, MO, USA). α-tubulin was used as a protein loading control. Secondary antibody is HRP-conjugated anti-rabbit (Jackson ImmunolResearch Labs, West Grove, PA, USA). Images shown in the figure were representative from 5 repeats. Densitometry of Western blots was quantified with NIH ImageJ software (Bethesda, MA, USA). The protein levels were first normalized to loading controls, and then normalized to experimental controls.

Bioinformatic analyses and luciferase-reporter activity assay
Targeting miRNAs for 3’-UTR of Beclin-1 mRNA were predicted using the following algorithms: TargetScan (https://www.targetscan.org) [24]. Luciferase-reporters were successfully constructed using molecular cloning technology. Target sequence for Bcl-2 miRNA 3’-UTR clone was purchased from Creative Biogene (Shirley, NY, USA). HULEC-miR-142a-5p, or HULEC-null, or HULEC-as-miR-142a-5p cells were seeded in 24-well plates for 24 hours, after which they were transfected with 1 μg of Luciferase-reporter plasmids per well using PEI Transfection Reagent. Luciferase activities were measured using the dual-luciferase reporter gene assay kit (Promega, Beijing, China), according to the manufacturer’s instructions.

Statistical analysis
All statistical analyses were carried out using the SPSS 18.0 statistical software package. All values are depicted as mean ± standard deviation and are considered significant if p < 0.05. All data were statistically analyzed using one-way ANOVA with a Bonferoni correction, followed by a Fisher’s exact test, as necessary.

Results
Isolation of mouse MSCs
Primary mouse MSCs were isolated and expanded in culture (Fig. 1A). After a clone was selected, differentiation assays were performed to confirm MSCs phenotype, including Von kossa staining to evaluate osteogenic induction, Oil red O staining to evaluate adipogenic induction and Alcian blue staining to evaluate chondrogenetic induction (Fig. 1B-D).

MSCs alleviate LPS-induced ALI in mouse lung
We then used 30 mice in our experiment. We transplanted MSCs into 10 mice via tail vein. One week later, 50 μg LPS from Escherichia coli in 40 μl PBS was given intratracheally to 20 mice, including 10 mice that had received MSCs. The other 10 mice received saline. We found that MSCs transplantation significantly reduced the LPS-induced increases in permeability index 6 hours after LPS administration (Fig. 2A). The histological patterns of lung with LPS-induced ALI were characterized by interstitial and intra-alveolar deposits of neutrophils and fibrin, prominence of alveolar macrophages, and intra-alveolar hemorrhage, and all these features were attenuated in the lung of the mice that had received MSCs (Fig. 2B).

MSCs increase autophagy of PECs after LPS-treatment
Since damages of PECs are the key process of LPS-mediated ALI pathology, we examined the survival of PECs by MSCs through autophagy. We isolated CD31+ PECs from dissociated mouse lung 8 days after LPS (Fig. 3A). Co-labeling for CD45 confirmed absence of CD45+ cell contamination in the CD31+ cell population (Fig. 3B). We found that MSCs significantly increased the LC3II vs LC3I levels in PECs, and the levels of key autophagy regulator, Beclin-1,
shown by representative Western blot (Fig. 3C), and by quantification (Fig. 3D). However, the mRNA levels of Beclin-1 were not altered in PECs by MSCs transplantation (Fig. 3E).
MSCs reduce miR-142a-5p, which suppresses Beclin-1 translation in PECs after LPS treatment

Since miRNAs often regulate translation of mRNA through binding to the 3'-UTR of mRNA of certain genes, we screened all Beclin-1-targeting miRNAs. Among these miRNAs, we found that miR-142a-5p targeted to 3'-UTR of Bcl-2 mRNA with the binding sites ranging from the 265th to 267th base site (Fig. 4A). In order to prove that this binding is functional, we modified miR-142a-5p levels in HULEC cells, and these HULEC-miR-142a-5p, HULEC-null (as a control) and HULEC-as-miR-142a-5p cells were confirmed of changes in miR-142a-5p levels (Fig. 4B). These cells were then transfected with 1μg of Beclin-1 3'-UTR luciferase-reporter plasmid. The changes in luciferase activities in these cells suggest that miR-142a-5p targets 3'UTR of Beclin-1 mRNA to inhibit its translation in PECs (Fig. 4C). Moreover, we found that miR-142a-5p levels in PECs in LPS-treated mice were significantly decreased by MSCs transplantation (Fig. 4D). Thus, MSCs reduces miR-142a-5p, which suppresses Beclin-1 translation in PECs after LPS treatment (Fig. 5).

Discussion

MSCs have been shown of highly immunosuppressive properties [8]. MSCs predominantly localized in the lungs when intravenously delivered, and they may secrete anti-inflammatory molecules that have local and distal effects [15]. Thus, pulmonary conditions could benefit from the immunosuppressive and anti-inflammatory activity of MSCs in the lungs. However, the effects of MSCs in the tissue repair are far more from immune regulation.

Here we isolated MSCs and confirmed phenotype of MSCs by differentiation assay. Besides differentiation assay, we have also checked CD70, CD90, CD105 and Sca-1 as positive
markers and CD34 as a negative marker. These data are not definite supporting data for a MSC phenotype, since all markers are not specific. Therefore, we did not show these data in the article. We found that MSCs transplantation significantly reduced the severity of LPS-induced ALI in mice. MSCs increased autophagy of PECs. Since autophagy is a process that often contradicts apoptosis, and leads to survival of the cells under harsh environment, these improvement of PEC autophagy may reduce apoptotic PEC cell death after LPS-induced lung injury and subsequently reduce the severity of blood vessel endothelial damages. These may reduce the infiltration of inflammatory cells and the production of pro-inflammatory cytokines. Although autophagy may also lead to cell death, which is called autophagic cell death, the coupling of autophagy and apoptosis pathways suggest that autophagy is a process...
that favors cell survival than apoptosis. Increases in autophagy often lead to decreases in apoptosis.

Then we found that MSCs increased Beclin-1 protein but not mRNA. These data suggest that the regulation of Beclin-1 by MSCs may be not at gene transcription, but at protein levels. The modulation of Beclin-1 protein could be either through alteration in protein translation by miRNAs, or through alteration in protein degradation via protein phosphorylation/de-phosphorylation, or sumoylation/de-sumoylation, or acetylation/de-acetylation, or ubiquitination. Although here we identified miR-142a-5p as a direct regulator of Beclin-1 protein translation, we do not exclude the possibility of modification of Beclin-1 protein levels through protein degradation or other mechanism. These possibilities may be examined in future studies.

Taken together, our data demonstrate that MSCs may alleviate LPS-ALI through downregulation of miR-142a-5p, which allows PECs to increase Beclin-1-mediated cell autophagy. Our results should provide new insights into using MSCs as a therapy for ALI.

Disclosure Statement

The authors have declared that no conflict of interest exists.

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