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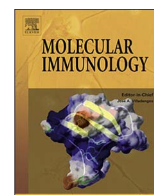

Research paper

BMSCs-derived miR-223-containing exosomes contribute to liver protection in experimental autoimmune hepatitis

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A B S T R A C T

Autoimmune hepatitis is a chronic inflammatory disease in the liver with potential to the development of liver fibrosis. Recent evidences suggest that bone marrow derived mesenchymal stem cells (BMSCs) may exert its therapeutic activity through exosomes. Moreover, miR-223 is highly expressed in BMSCs and plays an important role in autoimmune diseases. Therefore, in this study, hepatoprotective role of BMSCs and miR-223 was investigated in both mice and hepatocytes. Liver antigen S100 was used to establish autoimmune hepatitis model in mice while LPS and ATP were used to establish cell injury model in hepatocyte. Before the experiments, BMSCs were infected with pre-miR-223 and transfected with miR-223 inhibitor respectively. Exosomes from bone marrow stem cells were isolated by ultracentrifugation. Liver injury was evaluated by serum levels of ALT and AST as well as liver histology. Inflammation and cell death were examined by inflammatory cytokines and lactase dehydrogenase respectively. Both BMSCs-exo and BMSCs-exo$^{miR-223(+)}$ significantly reversed either S100 or LPS/ATP induced injury in mice and hepatocytes. Meanwhile, the expressions of cytokines, NLRP3 and caspase-1 were also downregulated by BMSCs-exo and BMSCs-exo$^{miR-223(+)}$ at both protein and mRNA levels in mice and hepatocytes. Moreover, BMSCs-exo$^{miR-223(-)}$ reverses the effects of BMSCs-exo and BMSCs-exo$^{miR-223(+)}$ in mouse AIH and in hepatocytes. In conclusion, bone marrow stem cell derived exosomes can protect liver injury in an experimental model of autoimmune hepatitis and the mechanism could be related to exosomal miR-223 regulation of NLRP3 and caspase-1.

1. Introduction

Autoimmune hepatitis (AIH) is one kind of autoimmune disorders and a chronic inflammatory disease of the liver, which is characterized by elevated serum aminotransferase levels, hyperglobulinemia, presence of non-species and no-organ-specific autoantibodies, progressive destruction of the liver parenchyma and development of liver fibrosis (Heneghan et al., 2013; Roberts et al., 1996). Although mechanism of AIH is still unclear, the pathogenesis may involve in genetic susceptibility, molecular and cellular mechanism and absence of immune regulatory function. The activation of immunocytes has been approved its relationship to hepatic damage, however, what is the specific mechanism, is ambiguous (Liberal et al., 2015). Moreover, there is limited treatment option and corticosteroid combined with azathioprine can only improve the survival rates to some extent. Although this kind of immunosuppressive treatments can prevent or reverse hepatic fibrosis,

Abbreviations: AIH, autoimmune hepatitis; EAH, experimental autoimmune hepatitis; BMSCs, bone marrow derived mesenchymal stem cells; miR 223, microRNA 223; LPS, lipopolysaccharide; ATP, adenosine triphosphate; ALT, alanine transaminase; AST, aspartate transaminase; NLRP3, NLR pyrin domain containing 3; ASC, apoptotic specklike protein containing CARD; EVs, extracellular vesicles; IL-6, interleukin 6; TNF-α, Tumor Necrosis Factor α; IL-1β, interleukin 1β; IL-17, interleukin 17; PBS, phosphate-buffered saline; DMEM, Dulbecco modified Eagle medium; ITS, insulin, transferrin and selenium; CM, culture medium; BSA, bovine serum albumin; H&E, hematoxylin and eosin; RIPA, radio immunoprecipitation assay; PMSF, phenylmethanesulfonyl fluoride; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; PCR, polymerase chain reaction; LDH, lactate dehydrogenase; ANOVA, analysis of variance; CAPS, cryopyrin-associated periodic syndrome; FMF, Familial Mediterranean fever

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its anti-fibrosis effects are inconsistent secondary gains (Czaja, 2014; Mistilis et al., 1968). Thus, the development of better therapies to directly prevent hepatic damage or fibrosis is emergent.

In recent years, mesenchymal stem cells (MSCs) based therapy has been extensively investigated in the area of tissue or organ protection. MSCs are considered as cells to differentiate into different cell types in the past but now they are more researches focused on their interaction with and influence other cells especially researches on communication within cells. Communication through extracellular vesicles (EVs) is getting more interests recently. There are many different types of secreted EVs with variation in their size, cell of origin, biogenesis, content and physiological setting for their release. Exosomes are lipid bilayer membrane vesicles, smaller than 150 nm in diameter, which are secreted by almost all kinds of cells and can directly transfer various bioactive molecules including mRNAs, microRNAs and proteins from donate cells to recipient cells (Tkach and Thery, 2016). Exosomes can be isolated in several ways while characterizing them is based on electron microscopy and the identification of its marker proteins. Their functions can expand to immunology neurobiology, stem cell and tumor biology. Their applications in clinic as biomarkers or therapeutic tools are the hottest research area (Bobrie et al., 2011). Moreover, the potential therapeutic application of exosomes in liver diseases has been reported in correction of metabolic deficiencies, promotion of liver regeneration, prevention of caner progression, and improvement of liver function (Maji et al., 2016; Yan et al., 2017; Lou et al., 2015; Tan et al., 2014).

MicroRNAs (miRNAs) are an important component of exosome and have attracted researchers' attention these years. The miRNAs, single-stranded and endogenous non-coding small RNAs that range from 18 to 25 nucleotides (nt) in length (Chen et al., 2016), regulate the expression of specific target gene. Recent investigations of miRNA expression profiles indicate that miRNAs have important roles in some autoimmune diseases. Among the most highly expressed miRNAs in animal bone marrow-derived mesenchymal stem cells (BMSCs) (Taibi et al., 1842; Haneklaus et al., 2013), the miRNA miR-223 is essential in the development and homeostasis of the immune system. Numerous findings have indicated that miR-223 can negatively regulate the expression of many inflammatory genes, such as IL-6 and NLRP3 (Haneklaus et al., 2012; Bauernfeind et al., 2012).

In this study, we employed an EAH model to examine potential of exosome with miR-223 from mouse BMSCs as therapeutic tool to protect liver injury.

2. Materials and methods

2.1. Murine experimental autoimmune hepatitis induced by hepatic S100 injection

Wild-type (WT) male C57BL/6 mice were purchased from the Shanghai Laboratory Animal Center (Shanghai, China) and maintained under specific pathogen-free conditions. All mice had free access to standard laboratory water and chow. Mice with ages of 4–6 weeks were employed for this experiment. For hepatic S100 preparation, ten mice were killed and liver antigens S100 were prepared freshly after perfusion of livers with phosphate-buffered saline (PBS) as previously described (Lohse et al., 1998). Briefly, the liver was minced and homogenized with cold PBS on ice and subsequently centrifuged at 150*g* for 10 min. Next, supernatants were further centrifuged an 100,000*g* for 1 h, and resulting supernatants was called S100 (Lohse et al., 1990), which was used for further separation by concentrating to 5 ml using an Amicon Ultra-15 filter (Millipore, USA) and then passing through a 90 cm CL–6 B Sepharose column (Pharmacia, Freiburg). There were three protein peaks collected from the column with the peak 2 was toxic components and the peak 1 and 3 were safe components as liver antigen. In this experiment, the peak 1 components with the concentration of 0.5–2.0 g/L were used. Moreover, for immunization, the liver S100 antigen was emulsified in an equal volume of complete Freund's adjuvant (Solarbio, China). Mice were injected intraperitoneally of this mixture on day 1 and a repeat injection on day 7. To evaluate the disease severity, three mice were sacrificed for histology and blood biochemistry assay. During the experiment, ten mice died.

2.2. Mesenchymal stem cell prepared and cell culture

BMSCs (P6), isolated from the femurs and tibias of C57BL/6 mice, were purchased from Cyagen Biosciences Inc. (Cyagen Biosciences, Guangzhou, China). The BMSCs phenotypic properties were performed by flow cytometry analysis. Cyagen Biosciences Inc provided identification results as: CD29 99.97%, CD44 99.32%, CD34 98.48%, Sca-1 96.85% and CD117 1.77%. Cells were cultured in minimum essential medium-alpha (α-MEM, Hyclone, USA) and incubated at 37 °C and 5% CO_2.

AML12 is a cell line established from hepatocytes from a male mouse and was purchased from American Type Culture Collection (Manassas, VA, USA). AML12 cells were maintained in DMEM/F12 (Gibco, USA) medium supplemented with 10% fetal bovine serum (Gibco), 1% ITS (Sigma), 40 ng/ml dexamethasone and 1% penicillin streptomycin mixture. For the *in vitro* experiment, AML12 cells were plated in 96-well plates at 1×10^4 cells per well or in 6-well plates at 1×10^5 cells. The cells were attached to bottom of well after 4–6 h and CM or LPS-CM was added. After a further incubation for 12 h at 37 °C, all medium was discarded, and then 5 mM ATP-CM (Sigma) was added to the well and incubated with cells for 6 h. In *in vitro* experiment, LPS/ATP-treated AML12 cells were incubated with the control medium, BMSCs-exo (20 μg/ml), BMSCs-exo$^{miR-223(+)}$ (20 μg/ml), and BMSCs-exo$^{miR-223(-)}$ (20 μg/ml).

2.3. miRNA precursor and miRNA inhibitor transfection

Lentivirus-encoding pre-miR-223 (LV-mmu-miR-223) and control lentivirus (Hu6-MCS-Ubiquitin-EGFP-IRES-puromycin) were obtained from Genechem (Shanghai, China). The synthetic miR-223 inhibitor was obtained from Ribobio (Guangdong, China). BMSCs (P6) at 80% confluency were infected with LV-miR-223, and control lentivirus according to the manufacturer's procedures. The synthetic miR-223 inhibitor was transfected into BMSCs (P8) using Lipofectamine 2000 when the cells achieved 90% confluence. After transfection, the supernatants were discarded and the cells were cultured with FBS-free medium for 24 h, and then exosomes were isolated. Then we got four BMSCs cell lines identified as: BMSCs, BMSCs$^{miR-223(+)}$, BMSCs$^{miR-223(-)}$ and BMSCs$^{miR-223(null)}$.

2.4. Isolation and characterization of exosomes

Isolation and purification of exosomes were performed as previously described (Thery et al., 2006). Briefly, MSCs culture medium was collected after replacing with serum-free medium and centrifuged at 300*g* for 10 min and then for an additional 10 min at 2000 g to remove dead cells. To remove cells debris, the supernatants were subjected to centrifuge at 10,000 g for 30 min. The final supernatants were ultracentrifuged at 100,000*g* for 1 h at 4 °C. The resulting pellets were resuspended in PBS and ultracentrifuged at 100,000*g* for another 1 h at 4 °C. Last, the final pellets were resuspended in 200ul PBS. Finally we got 4 kinds of exosomes and 3 of them, named as BMSCs-exo, BMSCs-exo$^{miR-223(+)}$ and BMSCs-exo$^{miR-223(-)}$, were used to validate their role in AIH or injured hepatocytes.

For exosomes characterization, we used western blot to detect the expression of TSG101, CD63, CD9 CD81 and Cytochrome c, as well as the ultrastructure of exosomes was observed under electron microscopy as previously described (Thery et al., 2006).

For exosomes uptake experiment, purified BMSCs-Exo was labeled with PKH67 Green Fluorescent Cell Linker Mini Kit (sigma) as

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