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Research Paper

Extracellular Vesicles Secreted by Human Adipose-derived Stem Cells (hASCs) Improve Survival Rate of Rats with Acute Liver Failure by Releasing lncRNA H19

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ABSTRACT

It has previously been reported that human adipose-derived stem cells (hASCs) can promote the regeneration of damaged tissues in rats with liver failure through a 'paracrine effect'. Here we demonstrate a therapeutic effect of hASCs derived Extracellular Vesicles (EVs) on rat models with acute liver failure, as shown by the improvement of the survival rate by N70% compared to controls. Gene sequencing of rat liver revealed an increase in human long-chain non-coding RNA (lncRNA) H19 after hASC-derived EVs transplantation. When the H19 coding sequence was silenced in hASCs and EVs were then collected for treatment of rats with liver failure, we saw a decrease in the survival rate to 40%, compared to treatment with EVs generated from non-silenced hASCs. These data indicate that lncRNA H19 may be a potential therapeutic target for the treatment of liver failure.

Research in context

Our research shows a therapeutic effect of hASCs derived Extracellular Vesicles (EVs) on rat models with acute liver failure, as demonstrated by a survival rate improvement of N70%. We speculate that hASCs derived Extracellular Vesicles promote hepatocytes' proliferation and improve the survival of rats with acute liver failure as a result of lncRNA H19 release.

1. Introduction

Liver failure poses a serious threat to the health of human beings. Orthotopic liver transplantation is currently the most effective treatment, but it is expensive and largely limited by the sources of the liver donors, making it unsuitable to serve as a common treatment method for the patients with end-stage liver failure ([1, 2]; Hessheimer et al., 2017). It has been shown that stem cells can promote the regeneration of impaired organs, tissues and cells through 'cell replacement' or the 'paracrine effect' ([4, 5, 53]; Jinglin et al., 2017; [7, 45]). As one of the adult stem cells, human adipose-derived stem cells (hASCs) are characterized by a high multiplication rate, broad differentiation potency and relatively easy accessibility, for which they are expected to be ideal seed cells for the treatment of some severe liver diseases such as acute liver failure (ALF) [8–10].

Originally, researchers held the view that stem cells repaired tissue injuries mainly through 'cell replacement' [11–13]; however, recent animal experiments have implicated that exogenous stem cells are largely eliminated by the recipient's immune system after a short period of time following in vivo transplantation, and very few stem cells differentiated into the functional somatic cells at targeted organs, suggesting that the therapeutic effect of transplanted stem cells may be attributable to their paracrine capacity [14–16]. Our findings in acute liver failure rat models show that hASCs facilitate the improvement of the rats' survival rate and liver function recovery, but the hepatocyte-related protein is not expressed within a short period of time after hASCs transplantation; as such, it can be predicted that the main active mechanism of hASCs may result from the 'paracrine effect' rather than 'cell replacement' [4, 53].

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HASCs can secrete proteins and RNA in the form of Extracellular Vesicles (EVs); the double-membrane structure of EVs can protect the internal protein and RNA from being degraded by various extracellular enzymes, and their functions can be exerted through interaction with the target cell surface receptors [17–20]. It has been reported that EVs isolated from BMSCs demonstrate better therapeutic effect in treating some heart and renal diseases in animal models than stem cells by promoting tissue repair and regeneration [21–28]. The efficacy of stem cell-derived EVs in treating acute liver injuries has also been reported [29, 30], however, the detailed mechanism remains unclear. In this study we obtained high-purity EVs from human adipose-derived stem cells. These EVs were then transplanted via the iliac vein into rats with acute liver failure, and a substantial therapeutic effect of hASCs-derived EVs was observed. We further explored the possible mechanism of hASCs-derived EVs in improving the outcomes of rats with acute liver failure through whole-genome RNA sequencing and bioinformatic analysis. We identified lncRNA H19, which was released by hASC-EVs, as a main target to exert the therapeutic effect by promoting hepatocytes' proliferation and viability of the rats with acute liver failure.

2. Materials and Methods

2.1. Isolation and Identification of hASCs

Adipose tissue was collected by liposuction from young healthy donors, which was approved by the ethical committee of the People's Liberation Army No. 85 Hospital, Shanghai, P.R. China (review serial number NO.2013/18). Relevant informed consent forms were signed by each donor before specimen collection. Adipose tissue was pulverized and cut into small pieces of about 1 mm^3 after being washed three times in phosphate buffer solution (PBS) (Gibco, USA), and 0.1% collagenase I (Gibco, USA) was added for 30-minute digestion. A complete culture medium of an equal volume (DMEM-F12 culture medium (Hyclone, USA), 10% fetal calf serum (Gibco, USA)) was added to terminate digestion, and centrifugation was performed for 10 min at a rate of 1000 rpm. PBS solution was used to re-suspend the cells, which were then seeded into a T25 cell culture flask at a density of 1×10^6/ml after filtration through a 40 μm cell strainer and placed into a 37 °C and 5% CO_2 constant temperature incubator (Thermo, USA) for culture.

Surface molecular markers of hASCs were detected by flow cytometry as follows: hASCs of 2nd passage were collected into 3 tubes of 500 μl single-cell suspension, add 2 μl of anti-human CD44-FITC, CD90-FITC, CD73-FITC, CD19-FITC, CD34-FITC, CD45-FITC and CD105-PE monoclonal antibodies (eBioscience, USA) respectively, wash in PBS three times after a 30-minute incubation at 37 °C and use a flow cytometer (caliber, BD, USA) for detection.

2.2. Adipogenic/Osteogenic/Chondrogenic Differentiation

The P2 generation hASCs were seeded into six-well plates at a density of $5 \times 10^3/cm^2$ and cultured for three weeks in adipogenic/osteogenic/chondrogenic differentiation culture media. hASCs were washed in PBS for three times, fixed with 4% formalin for 10 min, incubated for 30 min with Oil Red O (Sigma, USA) or for 3 h with Alizarin bordeaux (Sigma, USA) or for 3 h with Alcian blue (HUXMA-90041; Cyagen Biosciences Inc.) at room temperature, and observed under a microscope after being washed in PBS for three times.

2.3. Isolation of hASCs-derived EVs

Culture medium of hASCs was collected and centrifuged at 3000 ×g for 15 min to remove cells and cell debris. The supernatant was transferred to a sterile vessel and an appropriate volume of ExoQuick-TC (System Biosciences, USA) was added to the biofluid and mixed by inverting or flicking the tube. The sample was kept in refrigerate overnight at 4 °C and centrifuged again at 1500g for 30 min next day. EVs were spinned down by centrifugating at 1500g for 5 min. All the supernant was discarded via aspiration. EV pellet was resuspended in 500 μl using PBS.

2.4. Characterization of hASCs-derived EVs

Firstly, we detected the physical shape of EVs using scanning electron microscope. One drop of hASCs-derived EVs (about 10 μg) was added onto the sealing membrane and forceps were used to cover the fluid drop with a carbon support film copper grid for five to ten minutes; then one drop of 2% phosphotungstic acid staining solution was added onto the sealing membrane and the copper grid was transferred from the drop of hASCs-derived EVs to the drop of phosphotungstic acid; after stained for three minutes, filter paper was used to absorb the liquid, the hASCs-derived EVs were baked under a filament lamp and observed with a transmission electron microscope.

The next, we detected the concentration and purification of EVs using Nanosight granulometer. The sample of hASCs-derived EVs was prepared in 1 ml of solution of a proper concentration. A Nanosight granulometer (NS300, UK) was used, the samples were injected into the sample cell, the suspension of hASCs-derived EVs was irradiated through the laser light source, the Brownian movement of the hASCs-derived EVs was observed with scattered light and the particle size distribution information of the hASCs-derived EVs was obtained. Then three-dimensional maps of the particle size were showed, corresponding quantitative distribution intensity and scattering intensity.

Then we used the Antibody Chip Reagent Kit (System Bioscience, USA) to test the surface markers of EVs. According to the instructions, the, 500 μg of hASCs-derived EVs protein was added to 500 μl of lysate, vortex mixing was performed for 15 s, 9 ml of EABB was added and mixed up and down for three times. 10 ml of the above mixture was added to the antibody chip and cultured overnight on a rocker at 4 °C. The mixture was abandoned, 10 ml of washing solution was added and kept stand on a rocker for 5 min at room temperature, the liquid was abandon, 10 ml of antibody mixture was added to the chip and kept stand on a rocker for 2 h at room temperature. The liquid was aspirated from the chip using a sheet of dry aspiration paper after washed for three times, mixed according to a 1:1 ratio so as to moderately develop the color of the liquid drops on the chips, and photos were taken under the chemiluminescence imaging system (LAS 4000 mini, GE, Japan).

The protein concentration of the EVs could be determined using BCA Protein Assay kit (Solarbio, Beijing, China). According to the instructions, the standard curve was plotted, the multi-gradient dilution of the hASCs-derived EVs were performed and seeded into a 96-well plate, 200 ml of BCA working solution was added to each well, the plate was kept stand for 15 to 30 min at 37 °C, and a multi-functional microplate reader (SoftMax Pro5, Molecular Devices, USA) was used to determine the absorbance value at a wavelength of A562 nm. The protein concentration was calculated according to the standard curve and sample data. The protein concentration of the EVs used in the subsequent tests was determined using this kit.

The amount of EVs could also be calculated by the expression of CD63 protein on surface of EVs. According to the instructions of the CD63 ELISA kit, 50 μl of protein standard and hASCs-derived EVs were added to a 96-well plate, and incubation was perform for two hours at 37 °C. After washed three times in a washing solution, 50 μl of EVs primary antibody was added and kept stand on a rocker for 1 h at room temperature. After washed three times in a washing solution again, 50 μl of EVs secondary antibody was added and kept stand on a rocker for 1 h at room temperature. After a third round of washing three times in a washing solution, 50 μl of TMB substrate was added and kept stand on a rocker for 30 min at room temperature; then 50 μl of stop buffer was added and the absorbance was detected using a multi-functional microplate reader when the color in the well turns from blue to yellow.

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