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Adipose Stem Cell-Derived Exosomes Alleviate the Process of Hypertrophic Scar

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1. Abstract

Hypertrophic scar is an excessive wound healing process for which there is no more satisfactory treatment. Adipose Stem Cell-Derived exosomes (ADSC-Exos) produce a good preventive effect in the hypertrophic scar. However, the therapeutic effect of ADSC-Exos on hypertrophic scar has not been clarified. In order to investigate whether adipose stem cell-derived exosomes have a therapeutic effect on established hypertrophic scar, primary Hyperplastic Scar-derived Fibroblasts (HSFs) were extracted and cultured with ADSC-Exos secreted by adipose stem cells (ADSCs). Under these conditions, ADSC-Exos significantly reduced the deposition of collagen I and III and α-Smooth Muscle Actin (α-SMA). Thus, we have found a new key factor to provide a new therapeutic option for hypertrophic scar.

2. Introduction

When deep skin injury is caused by operation, severe burn or skin puncture, HS is easily formed in the injury area [1]. Studies have shown that the incidence of HS after burn is 44%, and surgical trauma is more than 70% [2, 3]. Pathologically, HS is a pathological scar characterized by excessive growth of fibroblasts and excessive secretion of extracellular matrix. It is characterized by swelling of local scar tissue, which can be red or dark red, often accompanied by pruritus, pain and other symptoms [4]. If it occurs in the joint, which may cause joint movement disorder [5]. Histologically, HS showed thickening of epidermis and dermis, proliferation of keratinocytes and dermis, and disorder of collagen fibers [6]. HS not only affects the appearance and function of patients, but also easily leads to mental complex [7]. At present, the treatment of HS is divided into surgical treatment and non-surgical treatment, mainly including local injection of 5-fluorouracil, pressurized excipients, laser, radiotherapy and so on, which is not ideal [8]. Therefore, to find a safe and effective treatment of HS is the focus of this study.

Pluripotent Mesenchymal Stem Cells (MSCs) have been widely studied because they can repair damaged tissues and differentiate into multiple cells to restore the function of damaged parts. Based on the characteristics of mesenchymal stem cells, some studies have isolated ADSCs for the treatment of fibrosis [9-12]. Studies have shown that ADSCs can inhibit the activation of hepatic stellate cells, reduce collagen deposition, and improve the therapeutic effect of liver fibrosis [13-15]. A number of experimental studies have shown that ADSCs and their derivatives have significance in preventing scar formation [16, 17]. However, these cell-based therapies have many limitations, including immune rejection, tumor formation and so on. In order to solve these limitations, new acellular therapies, such as exosomes, have increasingly become the focus of research, which may provide a new therapeutic strategy for stem cell therapy.

Exosomes, double membrane vesicles with a diameter of 30-150 nm released from cells, have attracted extensive attention as acellular therapy in recent years [18]. ADSC-Exos is no less effective than stem cells. Studies have shown that ADSC-Exos can effectively inhibit liver fibrosis and improve liver function [19]. In interstitial pneumonia, ADSC-Exos can effectively prevent the further aggravation of pulmonary fibrosis, which provides a new choice for the treatment of fibrosis [20]. Therefore, ADSC-Exos is considered to be more safe and effective treatment. At the same time, the distribution of adipose tissue is relatively rich, and liposuction is less invasive. It is convenient and fast to obtain ADSC-Exos from adipose tissue, reduce the cost of treatment, and provide patients with more safe and reliable treatment options.

Therefore, this study intends to investigate the effect of ADSC-Exos on the fibrosis of hypertrophic scar, and provide a new option for the treatment of hyperplastic scarring.

3. Materials and Methods

3.1. Isolation and Characterization of ADSCs

Isolation of ADSCs was performed according to our previous report. Briefly, inguinal subcutaneous fat was collected from adipose tissue and minced into pieces less than 1 mm in diameter before digestion with 0.2% type I collagenase (Gibco, New York, USA) at 37 °C for 30 min. Afterwards, the digested sample was neutralized by DMEM containing 10% FBS and passed through a 70-μm filter to remove undigested tissue. The cells were collected and seeded in T-25 flasks at a final concentration of 5×10^6 cells/ml. The cells were cultured in a 37 °C incubator supplied with 5% CO_2. The cell culture medium was changed every two days. Fourth- to fifth-generation cells were used for subsequent experiments. We identified ADSCs through immunofluorescence analysis and oil red staining analysis in our study.

3.2. Isolation and Characterization of ADSC-Exos

Isolation and identification of ADSC-Exos was performed according to a previous report. Briefly, ADSC-conditioned medium was collected, and the cells were removed by 300 × g centrifugation for 10 minutes, 2000 × g for 20 minutes and 10000 × g for 30 minutes and then filtered with a 0.22 μm filter. The medium was centrifuged at 4 °C at 100000 × g for 70 minutes, and the supernatant was removed and leveled. Finally, the culture medium was centrifuged at 100000 × g at 4℃ for 70 minutes (Figure S2a). ADSC-Exos were collected from the bottom of the tube and then suspended in PBS for further analysis. The morphology of the exosomes was evaluated by transmission electron microscopy (TEM; EM902A, Carl Zeiss Microimage GmbH, Germany). The size and relative strength of ADSC-Exos were quantified by a NanoSight NS300 (Malvern Instruments Co., Ltd.) (Figure S2b-c). The expression of CD81 and TSG101 was detected by western blotting (Figure S2d). Next, the protein concentration was measured by a BCA protein assay kit.

3.3. Isolation and Characterization of Primary Hypertrophic Scar Fibroblasts (HSFs)

Isolation and identification of HSFs were performed according to a previous report. Briefly, the dermal portions of hypertrophic scar tissues were minced and cultured with tissue block explants to isolate HS-derived fibroblasts (HSFs). HSFs were cultured with DMEM (Gibco, NewYork, USA) with 10% FBS (Corning, USA) and 100 U/ml penicillin in a humidified incubator containing 5% (v/v) CO2 at 37 °C. Cells at passages three to five were used for subsequent experiments. The fibroblast surface antibody vimentin (1:200, Abcam, Cambridge, UK) was identified by immunofluorescence (Figure S1b-c).

3.4. In Vitro ADSC-Exo Treatment

HSFs were cultured in 6-well plates (2×10^4 cells/well). After the cells adhered, ADSC-Exos at different concentrations were added to the plate. ADSC-Exos and HSFs were cultured for 48 h. To determine whether the effect of ADSC-Exos is dose-dependent, we detected ADSC-Exos group and control. We compared these groups with the control group for subsequent experiments (Figure S1d-e).

3.5. Proliferation Assay

HSFs were inoculated in 96-well plates (3000 cells/well). ADSC-Exos were added to HSFs and observed for 0, 6, 12 and 24 h. Then, CCK-8 (10 μL/well) solution was added to each well to determine the cell proliferation rate at different times. We used a Multiskan FC system (Thermo Fisher Science, Inc., Waltham, MA) to measure the Optical Density (OD) at a wavelength of 562 nm.

3.6. Real-time qRT-PCR

The total RNA of treated HSFs was extracted with TRIzol to measure the concentration of RNA in different groups and stored at -80 °C. Then, according to the manufacturer's instructions, a one-step TBGreen ®PrimeScript RT–PCR Kit II (TaKaRa, Beijing, China) was used for quantitative RT-analysis. The qRT–PCR primers were as follows: GAPDH, forward:5′-GGAGCGACGATCCCTCCAAAAT- 3′ and reverse:5′-GGCTGTTGTCATACTTCTCATGG-3′; collagen Ⅰ, forward:5′-GTGCGATGACGTGATCGTGTGA- 3′ and reverse: 5′-CGGTGGTTTCATTGGTCGGT- 3′; collagen Ⅲ, forward: 5′-GCCAAATTATGTGTCTGTGACTCA- 3′ and reverse : 5′-GGGCGAGTAAGGAGCAGTTG -3′; α-SMA, forward:5′-GGCATTCAGCGAGACCACCTAC-3′ and re-

verse:5′-CGACATGACCGTTGTTGGCATAC-3′. qRT-PCR was performed using a LightCycler 480 real-time fluorescence PCR system. Finally, the expression of collagen Ⅰ, collagen Ⅲ and α-SMA was analysed.

3.7. Statistical Analysis

Student's t test was performed for statistical comparisons between the two groups. One-way analysis of variance (ANOVA) was performed to statistically compare differences among multiple groups. The significance level of all analyses was set at a P-value < 0.05.

4. Results

4. 1. Clinical Specimens Suggest α-SMA in HS was Increased

30 cases of 30-year-old normal skin tissues and 30 cases of HS tissues are collected for Immunohistochemistry staining. The results showed that the expression of α-SMA was increased (figure 1a-b). Statistical analysis showed that the expression of α-SMA was closely related to SEI. From these clinical results, we can conclude that α-SMA of HS was increased, which is consistent with previous reports, suggesting that we can treat HS by regulating Extracellular Matrix(ECM).

4.2. Characterization of ADSCs and HSFs

According to the Mesenchymal and Tissue Stem Cell Committee of the International Society for Cellular Therapy, we investigated the expression levels of cell surface markers. Immunofluorescence analysis showed that more than 98% of the cultured cells expressed CD73, PE-A and CD105, CD90 and a few cells expressed CD13, CD44 (figure 1c). This expression of cell surface markers is a characteristic protein expression of ADSCs. Adipose cells were verified by oil red O staining (figure 1d).

The HSFs suspension of P_2 was placed in 6-well climbing plate for 24 hours. After fixed cells climbing plate, the vimentin expression of fibroblasts was basically positive, while the control group was negative (figure 2a).

4.3. Purification, Isolation, and Characterization of ADSC-Exos

The exosomes were isolated from the supernatant of ADSCs (figure 2b) and identified by Transmission Electron Microscope (TEM), nano-flow cytometry and Western blot analysis. TEM analysis shows that ADSC-Exos are typical cup-shaped vesicle (figure 2c). Nano-flow analysis shows that the average diameter of ADSC-Exos is 30-150 nm (figure 2d), and the concentration is 1.36×10^{11} particles /ml. Western blot shows that the expression of target protein was different between exocrine and control cells, such as high expression of CD63 and TSG101 which indicate that we have successfully isolated ADSC-Exos (figure 2e).

4.4. Cytotoxicity of ADSC-Exos and its Anti-Fibrosis Effect in Vitro

In order to evaluate the effect of ADSC-Exos on HS, we first ADSC-Exos and HSFs were co cultured for 48h. The results showed that the proliferation of HSFs decreased in the ADSC-Exos group compared with control group (Figure 3a-b); CCK8 results showed that the OD value of HSF in ADSC-Exos treatment group was lower than that in the control group (Figure 3c). Therefore, ADSC-Exos inhibited the proliferation and migration of HSFs. In addition, Western blot results confirmed that ADSC-Exos could down regulate col1, col3 and α- SMA (P < 0.05) (Figure 3d). QRT PCR showed that with the increase of ADSC-Exos concentration, the mRNA expression of col1, col3 and α-SMA decreased indicates that ADSC-Exos can reduce the fibrosis of HS (Figure 3e). These results confirmed that ADSC-Exos could inhibit the biological function of HSFs and delay the fibrosis.

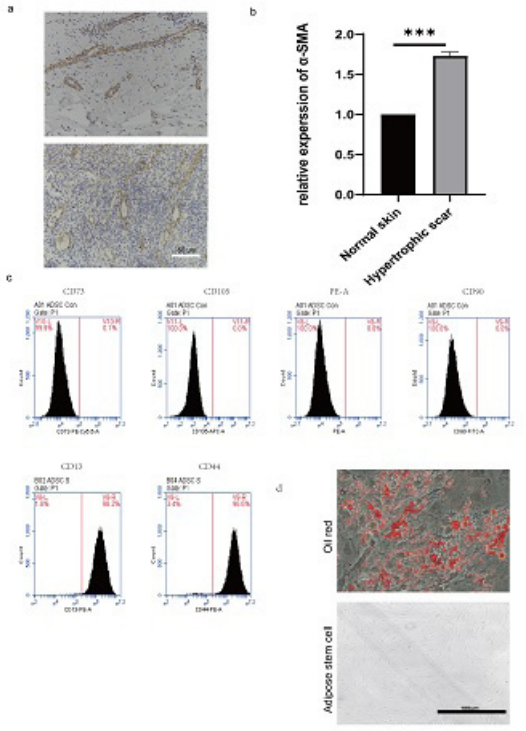

Figure 1: Characterization and identification of ADSC. (a): Representative immunohistochemistry of α-SMA expression in the scar tissue and normal skin; Scale bar,50 μm. (b): Representative quantification of α-SMA was shown (* * * P < 0.001). (c): Flow assay identification of adipose stem cell surface markers. (d): Characterization of adipose mesenchymal stem cells under optical microscope, Oil Red O.Scale bar,100μm.

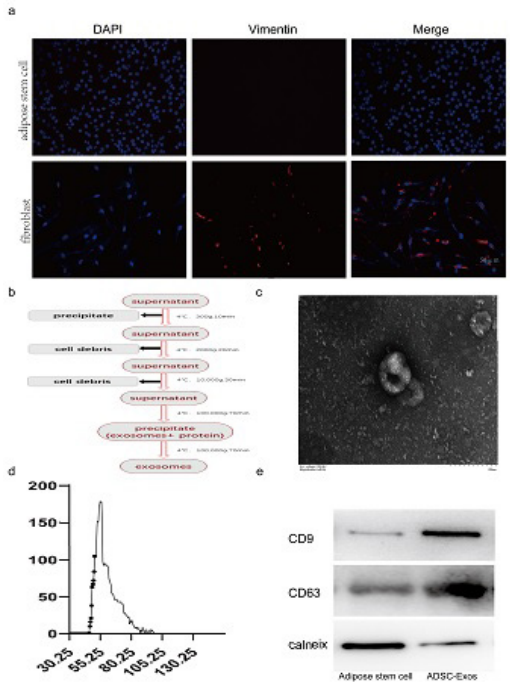

Figure 2: Characterization and identification of HSFs and ADSC-Exos. (a): Immunofluorescence analysis. Scale bar = 50μm. (b): Schematic diagram of preparation process of ADSC-Exos. (c): ADSC-Exos transmission electron microscope (TEM) images. (d): ADSC-Exos particle size distribution. (e): Western blot analysis of CD63 and TSG101 markers.

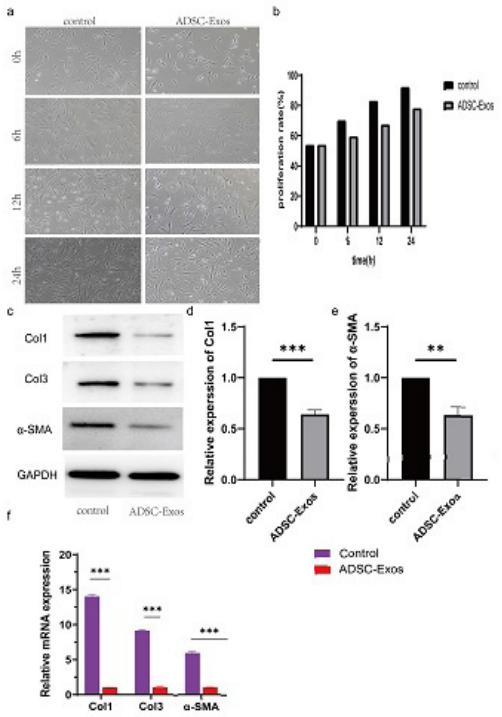

Figure 3: The effect of ADSC-Exos on HSFs proliferation and fibrosis. (a): The proliferation of HSFs was detected after ADSC-Exos intervention for 0 h,6h,12 h, and 24 h, respectively. PBS was set as a control. (b): The proliferation rate of HSFs after adding ADSC-Exos. (c): Western blot was used to observe the expression of collagen Ⅰ, collagen Ⅲ and α- SMA. (d-e): Representative expression of collagen Ⅲ, collagen Ⅰ, α-SMA. (f): qRT-PCR experiment was used to analyze the effects of ADSC-Exos co-cultured with HSFs on collagen Ⅰ, collagen Ⅲ and α- SMA changes in mRNA expression. (* * * P < 0.0001 * * P < 0.01 * P < 0.05 compared with control group)

5. Discussion

HS is a common skin fibrosis disease. Infection, large area deep burn and operation are the main factors for the formation of HS. Among them, fibroblasts are the main target cells of HS and the key to treatment. In recent years, there are more and more reports on the prevention and treatment of fibrosis, which also makes us have a clearer understanding of the process of fibrosis, and provides a new basis for inhibiting or even reversing the process of fibrosis. From the current experimental research, stem cell therapy is considered to be a promising solution. Experimental data show that the use of stem cells can speed up wound healing.

ADSCs have high yield, rapid proliferation and strong differentiation ability. They can differentiate into a variety of cells. When the body has an abnormal reaction, they can move to an appropriate position to generate other tissues and organs, and can also fill the defect and play the role of rapid repair. Therefore, ADSCs are considered as a promising stem cell therapy. Studies have shown that ADSCs are mesenchymal cells with pluripotent stem cells, which play their role through paracrine effect of a variety of cytokines. The interaction between cells is based on paracrine, connector or direct contact between cells. ADSCs can secrete growth factors that affect the biological function of skin fibroblasts, thereby inhibiting scar formation and photo aging. Although stem cell drugs have great clinical application value, many stem cell drugs also cause unexpected risks, such as tumor formation, cancer deformity, unnecessary immune response and the spread of uncertain factors. Therefore, acellular substances have become a research hot spot.

Exosomes,30-150 nm, are bilayer vesicles secreted by ADSCs and obtained after a series of centrifugations. It can regulate many cell-related pathological processes. In many research fields, exosomes are considered as intercellular communication. Exosomes carry non coding RNAs such as mRNA and miRNA, which affect the process of disease by regulating the expression of protein and RNA. In recent years, the role of exosomes in cell proliferation, migration and angiogenesis has been confirmed. However, there are few studies on ADSC-Exos in the treatment of HS. Therefore, this study demonstrated that ADSC-Exos can significantly reduce HS, and regulate the expression of col1, col3 and α- SMA in ECM inhibited HS. In vitro studies showed that ADSC-Exos was significantly absorbed by HSFs and produced a series of biological function changes. Cell proliferation and migration decreased in a dose-dependent manner. In addition, ADSC-Exos can adjust col1 and col3 and reduce the deposition of α-SMA. Our results suggest that ADSC-Exos is a promising cell-free therapy for HS.

In this experiment, we found that ADSC-Exos can inhibit HS. We speculate that these functions may mainly depend on some molecules of ADSC-Exos, such as microRNA, lncRNA and so on. In the future research, we will further explore the effect of molecules on HS.

6. Conclusion

ADSC-Exos are able to regulates ECM of hypertrophic scar fibroblasts, optimizes collagen synthesis and α- SMA deposition to treat HS. Therefore, ADSC-Exos may be a new cell-free expression vector for the treatment of HS in the future.

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