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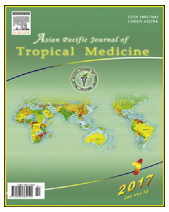

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Adipose tissue-derived stem cells ameliorates dermal fibrosis in a mouse model of scleroderma

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ABSTRACT

Objective: To investigate the therapeutic potential of adipose-derived stem cells (ADSCs) for limited cutaneous scleroderma (LS) in mouse models.

Methods: ADSCs were isolated from pathogen-free female C57BL/6 mice and LS was induced in wild type (WT) C57BL/6 mice via daily injection of bleomycin (0.1 mL × 300 μg/mL) for 4 weeks; then the ADSCs were subcutaneously injected into the dorsal area in the model treatment group, and 100 μL of phosphate-buffered saline (PBS) solution was injected into the same site in the model control group. Green fluorescent protein (GFP) was used to track the cells using an *in vivo* imaging system on days 7, 14, 21, and 28 after transplantation. All mice were sacrificed and histologic analyses were performed after 4 weeks, and the skin thickness, collagen deposition and the total content of hydroxyproline were evaluated. Additionally, immunohistochemistry were performed to compare the tissue expression and distribution of TGF-β1 and VEGF between the ADSCs treatment group and the treatment control group.

Results: WT C57BL/6 LS mouse model were successfully established and GFP *in vivo* fluorescence imaging showed that the translated ADSCs survived at the local for at least 4 weeks. Compared with the control group, the ADSCs treatment group significantly attenuated bleomycin-induced dermal fibrosis, reduced the skin thickness and the total content of hydroxyproline ($P < 0.05$). The ADSCs treatment group displayed significantly lower levels of TGF-β1 and higher levels of VEGF than the control group ($P < 0.05$).

Conclusions: ADSCs may provide a feasible and practical treatment for autoimmune diseases such as LS and ameliorate dermal fibrosis.

1. Introduction

Scleroderma is an autoimmune disease that is classified into the following two main subsets: limited cutaneous scleroderma (LS) and diffuse cutaneous scleroderma (DS). LS primarily affects the skin and is recognized to progress through two stages, an early inflammatory stage and a late fibrotic stage [1]. Currently, the commonly used immunosuppressant treatments lead to devastating long-term side effects and no established curative treatments for LS are available.

Stem cell transplantation is a relatively new therapeutic approach for the treatment of autoimmune and other diseases. Mesenchymal stem cells (MSCs) have been shown to possess immunomodulatory properties [2]. Therefore, MSCs transplantation is a promising therapy for reactivating the immune system to diminish fibrosis and restore the microvasculature for LS. Recent studies have identified adipose tissue as a new source of MSCs due to their abundance, ease of collection [3], and similarity to bone marrow stem cells (BMSCs) [4,5], including a comparable potential to differentiate into diverse cell lineages [6], such as endothelial cells [7]. Furthermore, ADSCs have been shown to potentially exert immunosuppressive effects [8]. Studies of

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human and murine ADSCs have documented their ability to release paracrine factors associated with suppression of the immune response [9,10].

In recent years, considerable attention has been paid on the immunomodulatory mechanisms of the reparative function of ADSCs. Our study aimed to evaluate the therapeutic potential of stem cells from adipose tissue in mouse models of LS and to determine the tissue expression and distribution of TGF-β1 and VEGF.

2. Materials and methods

2.1. Animals

A total of 5 pathogen-free female C57BL/6 mice (6 weeks of age, weighing 16–20 g) were presented by the Biological Treatment Center at Beijing Military Command Hospital. Forty wild type (WT) C57BL/6 mice were purchased from Beijing Laboratory Animal Center of Beijing Weitonglihua Company (Beijing, China). These WT C57BL/6 mice were randomly divided into five groups [group A: blank control group with normal mice, subjected to no disposal; group B: phosphate-buffered saline (PBS) model control group, subcutaneously injected PBS in the same dose as bleomycin; group C: bleomycin local injection to establish LS model; group D: model treatment group, bleomycin local injection to establish LS model and ADSCs subcutaneous transplantation to treat LS; group E: bleomycin local injection and PBS subcutaneous injection to set up model control], with 8 mice in each group. All mice were individually housed in separate clean rooms under controlled light and temperature (22 °C) conditions and provided with food and water *ad libitum*. The back skin of the mice was shaved, and the mice of group C, D, and E were subcutaneously injected with 300 μg/mL bleomycin in PBS (Nippon Kayaku Company, Tokyo, Japan) [11] and the mice of group B were injected only with PBS daily for 4 weeks. There wasn't any treatment to make for group A. Four weeks later, the back skin tissue of group A, B, and C were exsected, histological section was conducted, and the pathologic change was observed.

2.2. Cultivation of ADSCs

ADSCs were isolated from the 5 pathogen-free female C57BL/6 mice via a method based on cell density and adhesion, as described by Zuk *et al.* [12]. The cultured ADSCs at passages 3–4 were used in the subsequent *in vivo* studies.

Before the cells were injected into mice, they were analyzed for their capacity to proliferate and to differentiate toward adipogenic, osteogenic, chondrogenic, and myogenic lineages as described by Park IS *et al.* [13]. The ADSCs from passage 4 were plated in 96-well plates, and cell proliferation activity was evaluated after 1, 2, 3, 5, and 7 d using a MTT assay (C0009, Beyotime Biotechnology, Shanghai, China).

2.3. ADSCs local transplantation

The mice of group D received a single subcutaneous injection of ADSCs (2×10^6 cells in 100 μL of HBSS) in the back. The group E received a single subcutaneous injection of HBSS, 100 μL/only.

2.4. In vivo fluorescence imaging

The ADSCs express enhanced green fluorescent protein (GFP). *In vivo* fluorescence imaging was sequentially performed on anesthetized mice on days 7, 14, 21, and 28 after transplantation using an *in vivo* imaging system (IVIS, LB983NC100, Berthold, Germany). The survival of the transplanted ADSCs was observed by CCD camera.

2.5. Immunohistochemistry

The mice of group D, E were killed by the method of cervical dislocation 4 weeks later after the ADSCs treatment, the back skin tissue were exsected and divided into several parts to conduct hematoxylin–eosin (HE) coloration experiment, Masson coloration experiment and to detect the skin thickness and hydroxyproline content.

2.6. Skin thickness and collagen fiber analysis

The skin thickness was measured by Image-Pro Plus 6.1 under the electron microscope after the HE coloration and calculated from 5 parts averagely. Formalin-fixed sections were embedded in paraffin and stained with MT to examine the effects of bleomycin or ADSC treatment on the number of collagen fibers. Images were obtained using a Leica DM2000B + DFC295 light microscope (Germany) and were analyzed using Image-Pro Plus 6.1 software. The index of optical density (OD) for each group was calculated for the quantitative analysis and statistical comparison of collagen expression. The collagen fiber staining index of each specimen was calculated as the positively stained area × the staining intensity.

2.7. Hydroxyproline content

The total content of hydroxyproline were detected by using a photoelectric colorimeter according to the manufacturer's instructions (Nanjing Jiancheng Bioengineering Institute) and was calculated using the following equation: hydroxyproline content (μg/mg wet weight) = (sample OD − blank OD)/(standard OD − blank OD) × standard hydroxyproline content (5 μg/mL) × total volume of the hydrolyzate (10 mL)/tissue wet weight (mg).

2.8. Expression of TGF-β1 and VEGF

The samples were snap-frozen in liquid nitrogen, and 20 μm sections were prepared in a cryostat for immunohistochemical analysis. Finally, the sections were incubated with primary antibodies. The following primary antibodies were used: anti-TGF-β1 diluted 1:250 (ab92486, ABCAM, Cambridge, Britain) and anti-VEGF diluted 1:200 (ab52917, ABCAM, Cambridge, Britain). Digitalization was performed using a Pannoramic 250 FLASH digital slide scanner (3DHISTECH) and a CIS VCC-F52U25CL 3CCD progressive scan color camera (resolution: 0.37 μm/pixel). The H-Score, the sum of the percentage of positively stained cells multiplied by the weighted staining intensity, was calculated.

2.9. Statistics

All values are expressed as the mean ± SD. The group means were compared using an independent sample *t*-test with SPSS17.0 software. $P < 0.05$ was considered statistically significant difference.

3. Results

3.1. Characterization of ADSCs

The cultured ADSCs derived from the adipose tissue of GFP + transgenic mice showed a fibroblast-like morphology with central nuclei, long spindle, spiral shaped, and in alignment. The ADSCs labeled with fluorescent markers were similar with that under the optical microscope and were green fluorescent.

3.2. Capacity of ADSCs for proliferation and differentiation

The status of ADSC proliferation was observed to be in the incubation phase within 2 d after plating. From 3 to 5 d in culture, the cells were in the logarithmic phase, and cell proliferation plateaued from 5 to 7 d in culture. Subsequently, cell proliferation proceeded at a slower rate (Table 1). The plasticity of the ADSCs was assessed after lineage induction at passage 4 [14]. The ability of the cultured ADSCs to differentiate toward adipogenic, chondrogenic, and osteogenic lineages was determined based on the presence of lipid vacuoles, mucopolysaccharide-rich extracellular matrix proteins, and calcium deposits, respectively. Furthermore, the differentiation of ADSCs into adipogenic, chondrogenic, and osteogenic lineages was assessed via Oil Red O staining, Alcian Blue staining, and Alizarin Red staining, respectively. The results indicated that these cells were multipotent.

Table 1
GFP-rADSCs cell growth.

Time/d	1	2	3	4	5	6	7
OD	0.37	0.45	0.68	0.75	0.94	0.69	0.60

3.3. Induction of LS animal model

Approximately 1 week later after the induction, the skin began to lose its hair, locally thicken, and become less flexible. At the fourth weekend, the skin at the site of the injection with bleomycin was thickened and hardened and the hair was seriously lost. The skin of PBS control group did not change significantly in the skin.

MT staining demonstrated intense deposition of collagen in the dermis after bleomycin treatment compared with PBS treatment. MT staining showed that the density of collagen fibers was increased in parallel with the induction of LS. Furthermore, HE staining also showed an increased width of the dermis following bleomycin treatment. Cutaneous fibrosis was quantified by analyzing the hydroxyproline content and the collagen fiber staining index. The results showed that the hydroxyproline content in the punch-biopsied skin samples was increased after bleomycin treatment compared with PBS treatment.

The results of skin thickness and hydroxyproline content were shown in Table 2.

Table 2
The skin thickness and hydroxyproline content of group A, B, C, D, and E ($n = 8$).

Group	Skin thickness (μm)	Hydroxyproline content (μg/mg)
A	125.42 ± 7.16$^{*\#}$	2.63 ± 0.48$^{*\#}$
B	131.89 ± 2.44*	2.81 ± 0.46*
C	203.93 ± 20.39	7.13 ± 0.80
D	136.06 ± 4.49	3.80 ± 0.84
E	150.47 ± 5.16$^{\#}$	6.05 ± 0.78$^{\#}$

$^{*}P < 0.05$, compared with group C. $^{\#}P < 0.01$, compared with group D. Group A: Blank control group; B: PBS control group; C: Model observation group; D: Model treatment group; E: Model treatment control group.

3.4. Transplantation of ADSCs into mice with LS

According to fluorescence microscopic images captured on days 7, 14, 21, and 28 after transplantation, the ADSCs were localized to the injection site of the skin. A clear fluorescent signal was detectable in the subcutaneous tissue, and this result confirmed that the ADSCs survived for at least 4 weeks. At the fourth weekend, the site of the transplantation softened the skin significantly, and skin hair grew again. The skin of the control group did not change significantly in the skin, No viral complications or infections were detected. Using florescence microscopy, frozen skin sections were analyzed to validate the IVIS results. GFP fluorescence in the transplanted cells was easily detectable via direct fluorescence microscopy, and localized colonies of ADSCs were detected in the subcutaneous tissue 4 weeks after transplantation.

The result of skin thickness, hydroxyproline content and the collagen fiber staining index were shown in Tables 2 and 3.

Table 3
The collagen fiber staining index and the H-scores for VEGF and TGF-β1 of group A, D, and E ($n = 8$).

Group	Collagen fiber staining index	VEGF H-score	TGF-β H-score
A	224 256 ± 71 570*	119.2 ± 18.6	121.6 ± 17.2
D	460 653 ± 55 877	133.8 ± 26.6$^{\#\triangle}$	113.5 ± 23.7$^{\#\triangle}$
E	665 838 ± 142 431*	110.3 ± 12.8	143.2 ± 19.6$^{\#}$

$^{*}P < 0.05$, compared with group D; $^{\#}P < 0.05$, compared with group A; $^{\triangle}P < 0.05$, compared with group E.

3.5. Expression and distribution of TGF-β1 and VEGF

Immunohistochemical staining using the anti-TGF-β1 antibody revealed significantly lower TGF-β1 levels in the model treatment group than in the model control group ($P < 0.05$). The H-Scores for TGF-β1 and VEGF were shown as Table 3.

4. Discussion

Scleroderma is an autoimmune disease characterized by vascular injury, excessive accumulation of ECM in skin and

various internal organs, and immunological abnormalities [15]. Scleroderma is classified into the following two main subsets: LS and DS. In contrast to systemic sclerosis, LS does not affect the internal organs but primarily affects the skin; therefore, developing therapies to treat its skin-related symptoms has been the focus of many clinical trials. Recently, a few studies have investigated the use of cell therapy for scleroderma. Therefore, the authors evaluated the efficacy of a stem cell-based therapy in an LS animal model.

MSCs have been shown to possess immune regulatory capabilities, anti-inflammatory activities, and angiogenic potential. MSCs can secrete soluble factors such as IL-6, macrophage colony-stimulating factor [16], IFN-γ, TNF-α, and IL-10 [17]. Furthermore, MSCs have been shown to suppress the activation and proliferation of T and B lymphocytes and to interfere with the differentiation, maturation and function of dendritic cells. MSCs can also release anti-inflammatory and anti-apoptotic molecules and may therefore protect tissues against damage [18]. In addition, studies have confirmed that MSCs have low immunogenicity due to their low expression levels of major histocompatibility complex (MHC)-Ⅰ and lack of expression of MHC-Ⅱ and costimulatory molecules, including B7-1 (CD80), B7-2 (CD86), and CD40 [19]. MSC therapy has shown therapeutic potential for the treatment of many diseases, including systemic lupus erythematosus (SLE), rheumatoid arthritis (RA), multiple sclerosis (MS), and thyroiditis [20–22].

Studies have shown that ADSCs resemble BMSCs in terms of morphology, growth rate, and cell surface marker expression profile [23]. Additionally, ADSCs have the ability to differentiate into various lineages including adipocytes, osteoblasts, chondrocytes, myoblasts, and endothelial cells [24]. In recent years, increased interest has turned to the plasticity and therapeutic potential of ADSCs isolated from adipose tissue [25]. ADSCs can be collected from adipose tissue as a byproduct of liposuction in esthetic surgeries [26] and easily cultured; they also have a high capacity for rapid expansion *in vitro*, and can be less immunogenic and immunosuppressive than other cells, which show that they can be applied in allogeneic transplantation [27]. Recently, several groups reported the use of stem cells to treat scleroderma. Luis A. Ortiz *et al.* demonstrated that engraftment of murine MSCs into the lung reduced inflammation and collagen deposition in the lung tissue of mice challenged with bleomycin [28]. Manizheh Azhdari *et al.* determined the therapeutic potential of vascular derivatives of human-induced pluripotent stem cell-derived ECs (hiPSCs) in a scleroderma model [29]. Finally, Nicolò Scuderi *et al.* suggested that ADSCs are a potentially valuable source of cells for skin therapy in rare skin diseases including scleroderma [30].

The underlying pathogenesis of scleroderma involves a complex interplay of inflammation, fibrosis, and vasculopathy. VEGF is a central regulatory factor for the formation of new vessels that controls several steps of angiogenesis. Studies indicate that VEGF has protective effects in SSc patients [31]. TGF-β1 is known to be a potent stimulus of fibrosis in scleroderma, but ADSCs were shown to display the strongest immunosuppressive and angiogenic capacities [32]. Therefore, this study transplanted ADSCs into LS model mice and used an IVIS to trace the transplanted cells and confirmed that the GFP-labeled ADSCs were located in the subcutaneous tissue of LS model mice for up to 4 weeks after transplantation. It was showed that local injection of ADSCs improved the condition of the skin and prevented hyperplasia of collagen fibers in LS model mice. This study compared the tissue expression and distribution of TGF-β1 and VEGF. The model treatment group displayed significantly lower levels of TGF-β1 and higher levels of VEGF than the control group. These findings indicate the efficacy of the transplanted ADSCs in promoting skin repair. VEGF, in concert with TGF-β1, is a crucial factor in the pathogenesis of scleroderma [33]. In accordance with this result, Koch and Distler [34] showed increased production of VEGF in the skin of SS patients. The results demonstrated the antifibrotic and pro-angiogenic effects of local administration of ADSCs in a mouse model of BLM-induced LS.

The results of present study suggested that ADSCs lessened LS symptoms in the *in vivo* model studied. The ADSC-induced alterations in the expression of TGF-β1 and VEGF, which play a key role in the condition of the skin, prevented hyperplasia of the collagen fibers and may have promoted formation of blood vessels. Despite the promising potential of ADSCs for the treatment of LS, future studies are needed to investigate the mechanisms underlying the beneficial effects of ADSCs in LS for the purpose of adapting their use to clinical applications.

Conflict of interest statement

We declare that we have no conflict of interest.

Ethics and morals statement

The authors declare that the procedures were in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals (NIH Publications No. 8023, revised 1978). All the study has been approved by the Ethics Committee of General Hospital of Beijing Military Command.

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