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Transplantation of Human umbilical cord mesenchymal stem cells promotes functional recovery after spinal cord injury by blocking the expression of IL-7

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Abstract. – OBJECTIVE: This research aimed to investigate the therapeutic effects of transplanted human umbilical cord mesenchymal stem cells (hUCMSCs) on spinal cord injury in mice and to explore its molecular mechanism.

MATERIALS AND METHODS: Spinal cord injury model in C57BL/6J mice was established. On the 10th day of SCI, hUCMSCs were injected into the center of spinal cord injury area (hUCMSC), and control groups (Control) were injected with an equal amount of medium. Western blotting, Real Time-PCR, immunohistochemistry, and flow cytometry, were used to analyze the content of IL-7, inflammatory cytokines, and macrophages after spinal cord injury in different groups. Open field and Rota-Rod tests were used to determine the effect of hUCMSC transplantation on motor function recovery in SCI mice.

RESULTS: Compared with the control mice, hUCMSC transplantation therapy significantly improved the motor function, myelin, and nerve cell survival in spinal cord injury site in SCI mice. It also reduced the expression of IL-7, IFN-γ, and TNF-α in injured sites but increased IL-4 and IL-13 expression and promoted the activation of M2 macrophages at the site of injury.

CONCLUSIONS: Transplantation of hUCMSCs in SCI mice can promote the polarization of M2 macrophages by reducing the expression of IL-7 in the injured site, thereby weakening the inflammatory response at the injured site, promoting the repair of the injured site and improving the motor function.

Key Words:
hUCMSC, IL-7, Spinal cord injury, Inflammation.

Introduction

Spinal cord injury (SCI) is a serious traumatic central nervous system disease with varying degrees of neurological dysfunction. Improper or untimely treatment can lead to lifelong disability, even respiratory failure and death, often resulting in partial or complete paralysis of the limbs[1]. SCI is a complex pathophysiological process that can be cascaded. There are two mechanisms in the process of the development and progression of SCI[2]. One is the primary injury, which is the direct injury of the neuron caused by the strong mechanical external force at the time of injury, and the occurrence of cell necrosis and apoptosis in an extremely short time after injury. The other is the secondary injury, which is caused by inflammation, ischemia, lipid peroxidation, collagen scars, etc.[3,4]. The inflammatory responses caused by the early microcirculatory disturbance of SCI may play a crucial role in secondary SCI[5,6]. With the development of molecular biology, people have made great progress in the understanding and treatment of SCI. There are also many new methods for the treatment of SCI. Cell transplantation is currently a research hotspot. The cells currently used for experiments are olfactory ensheathing cells[7,8], Schwann cells[9,10], embryonic stem cells[11,12], neural stem cells[13], bone marrow mesenchymal stem cells[14], umbilical cord mesenchymal stem cells[15,16], etc.[17]. Compared with other cells, human umbilical cord mesenchymal stem cells are stem cells with a high degree of self-renewal and multi-pluripotency. They have ample sources, and are easy to be isolated, cultured and expanded, have low immunogenicity, and do not involve moral and ethical aspects, thus avoiding the disadvantages and deficiencies of other cells[18,19]. Wang et al[20] reported that human umbilical cord mesenchymal stem cells (hUCMSCs) combined with quercetin were used to treat SCI mice by reducing secondary damage and promoting functional recovery. Our previous studies[21] have confirmed high expression of interleukin-7

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(IL-7) at the site in early SCI, which mediates high-inflammation reaction at the site of injury, thereby inhibiting spinal cord nerve regeneration and motor function recovery. Using anti-IL-7Rα mono-clonal antibody (A7R34) to block IL-7 signaling contributes to the activation of M2 macrophages, thereby affecting the production of T helper (Th) 1 and Th2 cytokines, inhibiting the inflammatory response at the site of SCI, and promoting the repair of SCI and recovery of motor function. In this work, we established a SCI model in C57BL/6J mice and performed hUCMSC transplantation 10 days after SCI. After that, we assessed the effect of hUCMSC transplantation at both histopathological and behavioral levels. The levels of Th1 and Th2 cytokines, M1/M2 macrophage content, and IL-7 expression in SCI sites in mice were also examined to investigate the molecular mechanism of hUCMSC transplantation in SCI mice. In summary, we have found that transplantation of hUCMSCs to treat SCI mice can promote activation of M2 macrophages by inhibiting the expression of IL-7 at the site of SCI, which in turn reduces the inflammatory response and inflammatory plumpness at the site of injury and promotes repair of SCI site and recovery of motor function.

Materials and Methods

Ethics Committee Approval

Animal welfare and the relevant experiment were carried out in compliance with the guide for the care and use of laboratory animals. The study was approved by the Ethics Committee of Southwest Medical University Affiliated Hospital.

Experimental Animals and SCI Models

Adult C57BL/6J mice (half male and female) that have been adapted for feeding for one week (room temperature 20-24°C, day 12 h and night 12 h, air humidity 60%) were used. The mice were anaesthetized using chloral hydrate i.p. injection, in a prone position, using a modified Allen's hitter (Zenda Instruments Co., Ltd., Shanghai, China), performed a vertical (25 mm, 5 g mass) strike against the spinal cord of T10 segment, until the mouse's tail appeared convulsive swings, and both lower limbs and somatic retraction shook, indicating a successful SCI. After modeling, urination twice/d until normal urinary tract reflex was observed in model mice. Three days before transplantation until the end of the study, 10 mg/kg of cyclosporin A was subcutaneously injected (Registration No. H20050433, Novartis Pharma, Stein AG, Switzerland) once a day.

hUCMSC Isolation, Culture And Identification

Umbilical cord blood samples were collected at the Department of Obstetrics and Gynecology at Southwest Medical University Affiliated Hospital and were used for research after the mothers or their families were informed and signed the informed consent. Umbilical cord blood was harvested within 6 hours after harvest, followed by clip-pruning, washing with antibiotics containing phosphate-buffered saline (PBS) (135 mM NaCl, 2.7 mM KCl, 2.0 mM KH2PO4, 8 mM Na2HPO4, 1% Penicillin-Streptomycin Solution, pH=7.2) and block method to isolate hUCMSC. The detailed methods were as follows: the umbilical vein was dissected longitudinally, and the intima was peeled. The remaining lumen was cut to a size of 3.0-5.0 mm^3 and seeded into a T25 flask (707003, NEST, Hongkong, China). The flask was put into an incubator under 37°C and 5% CO_2. Then, 1 mL of Dulbecco's Modified Eagle Medium (DMEM)/F12 medium (D6046, Sigma-Aldrich, St. Louis, MO, USA) with 2% FBS (10099-141, Gibco, CA, USA), 40% MCDB201 (M6770, Sigma-Aldrich, St. Louis, MO, USA), 10 μg/L of platelet derived growth factor (P5208, Sigma-Aldrich, St. Louis, MO, USA), 10 μg/L of basic fibroblast growth factor (S20020024, Beijing Double Heron Medicine, Beijing, China) was added daily into the flask. The medium was changed every 3 days, while the attached tissue blocks were removed after been cultured for 20 days. Identification: the second generation human umbilical cord mesenchymal stem cells were used to prepare a single cell suspension with a concentration of 3×10^6 /ml, a total of 13 tubes. We added 15 μl of mouse anti-human CD73-PE (562817), CD105-PE (560839), CD90-FITC (553013), CD34-PE (551387), CD45-PE (552833), CD14-FITC (562691), and HLA-DR-PerCP (560652) antibodies, respectively into 7 tubes out of the 13 tubes. The corresponding isotype control antibodies were added into rest 6 tubes. The cells with antibodies were incubated at 4°C for 30 min, and then washed twice with PBS, fixed with 4% paraformaldehyde, examined using a flow cytometer (LSR II, BD Pharmingen, (Franklin Lakes, NJ, USA). All the antibodies used were purchased at BD Pharmingen (Franklin Lakes, NJ, USA).

hUCMSC Transplantation for Treatment of SCI Model Mice

14 days after SCI model mice were successfully created, hUCMSC transplantation was performed. On the one hand, hUCMSC cells were cultured *in vitro* to 50% confluence using conventional serum-free DMEM/F12 medium; then, the medium was discarded and the cells with 10 mg/L bromodeoxyuridine nucleoside (ED1100, Wuhan Dr. De Biological, Wuhan, China) were cultured for 72 h; next, 0.25% trypsin-EDTA (25200-256, Gbico, CA, USA) was added dropwise to prepare for the single cell suspensions. On the other hand, 10% chloral hydrate was injected into the peritoneal cavity to anesthetize the mice. Each layer of the skin and adjacent tissues of the mouse were cut with a scalpel to fully expose the injured spinal segments. 10 μl of cell suspension containing 1×10^6 hUCMSCs was injected into the center of the lesion area with a microsyringe and sutured to the skin layer by layer.

Immunochemistry

SCI model mice were sacrificed by cervical dislocation. The left ventricle was perfused with 4% paraformaldehyde. The SCI area was dissected and revealed. The injury area was placed into a 30% sucrose solution, maintained in a 4°C incubator and set to a thickness of 16 μm and continuous sagittal frozen sections were made.

Luxol fast blue (LFB) staining: (1) the sections were washed with distilled water and then placed in a 0.1% LFB solution (L0294, Sigma-Aldrich, St. Louis, MO, USA) and sealed and staining at 60°C for 8-16 hours; (2) after washing with distilled water, 95% ethanol was added; (3) the color was separated with 0.05% aqueous solution of lithium carbonate for more than 10 seconds; (4) we continued to separate color with 70% ethanol until the gray and white matter were clearly observed under the microscope; (5) after washing with distilled water, counterstain with 10% tar purple solution plus several drops of glacial acetic acid dye solution for 10 minutes was added; (6) 70% ethanol split the stained color to the nucleus and nissl body to be red.

Immunofluorescence staining: (1) after rinsing, the sections were incubated with trion-x100 at room temperature for 1 hour; (2) neurofilaments (NF) immunofluorescence staining: anti-Neurofilament heavy polypeptide antibody (1:200, ab8972, Abcam, Cambridge, MA, USA) as a primary antibody was first incubated at 4°C for 72 h, then removed to room temperature for 20 min, and finally incubated at 37°C for 30 min. General immunofluorescence staining: PE rat anti-CD11b (1:500, 553311, BD Pharmingen, (Franklin Lakes, NJ, USA), fluorescein isothiocyanate (FITC) anti-Arginase 1 (1:500, 553164, BD Pharmingen, Franklin Lakes, NJ, USA); FITC mouse anti-inducible Nitric Oxide Synthase (iNOS) (1:1000, 610331, BD Pharmingen, Franklin Lakes, NJ, USA) were added and incubated overnight at 4°C; (3) For NF immunofluorescence staining, PE F(ab')2-Rabbit anti-Mouse IgG (H+L) (1:1000, PA1-84431, Invitrogen, Carlsbad, CA, USA) as secondary antibody was incubated at room temperature for 3 h.

Behavior Analysis

The open movement and Rota-rod treadmills were used to evaluate motor function after SCI injury[21]. The scale movement function score of Basso Mouse Scale for Locomotion (BMS) was used to assess the recovery of hindlimbs in mice[22]. The Rota-rod treadmill was used to evaluate motor coordination and fatigue resistance of mice. All tests were conducted for a period of 10 weeks, and each test was performed by two reviewers independently.

Western Blotting

After the mice were sacrificed, the injured tissue of spinal cord was taken and placed in a mortar. After the appropriate amount of liquid nitrogen was added, the tissue was quickly ground. After the tissue was thoroughly ground, the radioimmunoprecipitation assay (RIPA) lysate (Beyotime Biotechnology, China) was used to extract the total protein. The RIPA lysate-containing tissue was collected into an Eppendorf (EP) tube and pipetted repeatedly; then, it was placed on ice for 10 minutes and centrifuged at 12,000 rpm for 10 min to collected total proteins. 20% sodium dodecyl sulfate (SDS) buffer solution was added to the supernatant until the final concentration of SDS was 1%, and it was boiled at 100°C for 5 min. The protein concentration was measured by bicinchoninic acid (BCA) Protein Assay Kit (Beyotime Biotechnology, ShangHai, China). 75 μg of total proteins were loaded into each lane, separated by 12% SDS-polyacrylamide gel electrophoresis (PAGE) (90 V, 0.5 h; 120 V, 1 h) and transferred (400 mA, 1.5 h) to polyvinylidene difluoride (PVDF) film (Amersham Biosciences, Boston, MA, USA); then, it was fixed with methanol for 1 min, washed three times (5 min/each) with TBST (10 mM Tris-HCl,

Table I. RT-qPR primers.

Gene	sequence (5′-3′)
IL-7	F:CTGCTGCACATTTGTGGCTT
	R:AAGAAGACAGGGATGCGGTG
TNF-α	F:ACCCTCACACTCACAAACCA
	R:ATAGCAAATCGGCTGACGGT
IFN-γ	F:CCTCATGGCTGTTTCTGGCT
	R:TCCTTTTGCCAGTTCCTCCA
IL-13	F:TGCCATCTACAGGACCCAGA
	R:CTCATTAGAAGGGGCCGTGG
IL-4	F:GGATGACAACTAGCTGGGGG
	R:ATGGATGTGCCAAACGTCCT
GAPDH	F:GATGAACCTAAGCTGGGACCC
	R:TGTGAACGGATTTGGCCGTA

150 mM NaCl, 0.1% tween 20, pH = 7.6), and then 5% skimmed milk (prepared by TBST) was used as the blocking agent, and it was blocked for 1 hour at room temperature. IL-7 antibody (ab9732, 1:1000, Abcam, Cambridge, MA, USA) or β-actin antibody (ab8227, 1:2000, Abcam, Cambridge, MA, USA) diluted in 5% skim milk was added and incubated overnight at 4°C. After washing 3 times (10 minutes once) with TBS containing 0.05% Tween-20 (TBST), goat anti-rabbit secondary antibody (ab205718, 1:2000, Abcam, Cambridge, MA, USA) was added and incubated for 1 hour at room temperature. Then, enhanced chemiluminescent substrate (ECL) luminescence solution was added for detection. The expression of the target protein was analyzed by Image J software, and the relative expression of the target protein was characterized by the gray value of the target protein band/the gray value of the β-actin protein band.

RNA Extraction and Quantitative Real Time-PCR (qRT-PCR)

Total RNA was extracted from the injured spinal cord in mice by using the RNeasy kit (79254, Qiagen, BeiJing, China). The extracted RNA was used to synthesize cDNA by PrimeScript™ RT reagent kit with gDNA Eraser kit (RR047A, TaKaRa, Otsu, Shiga, Japan). The PCR parameters were set: 37°C for 15 minutes, 85°C for 5 s. RT-qPCR: 20 μl RT-qPCR system was prepared with the SYBR™ Green PCR Master Mix (4312704, Invitrogen, Carlsbad, CA, USA) and amplified by using ABI 7300 Fluorometric qPCR instrument (Applied Biosystems, Foster City, CA, USA). The PCR parameters were set: 95°C for 30 s, 90°C for 5 s, and 65°C for 30 s, 40 cycles. Using β-actin as an internal reference, the relative expression level of the target gene was calculated by using the $2^{-\cdot t \cdot t}$ method (Table I).

Flow Cytometry Analysis

After the mice were sacrificed, the tissue of injured spinal cord was taken, and then single cell suspension was prepared[21]. Cells obtained by gradient centrifugation were washed with PBS (135 mM NaCl, 2.7 mM KCl, 1.5 mM KH_2PO_4, 8 mM K_2HPO_4, adjusted to pH 7.2 with HCl and NaOH). The obtained cells were centrifuged and then incubated with Pacific Blue™ Rat Anti-Mouse CD45R (1:500, 558108, BD Pharmingen, Franklin Lakes, NJ, USA) or APC-CyTM7 Rat Anti-CD11b (1:500, 561039, BD Pharmingen, Franklin Lakes, NJ, USA) or APCTM anti-Gr-1 (1:500, 560599, BD Pharmingen, Franklin Lakes, NJ, USA) as primary antibodies for 30 min on ice. Cells were fixed and permeabilized for intracellular staining. After washed with PBS, FITC anti-Arginase 1 (1:500, 553164, BD Pharmingen, Franklin Lakes, NJ, USA), or FITC mouse anti-i-NOS (1:1000, 610331, BD Pharmingen, Franklin Lakes, NJ, USA) was added on ice for 30 min, and finally analyzed by flow cytometry.

Statistical Analysis

Data were statistically analyzed by SPSS 20.0 software package (Armonk, NY, USA). The data were expressed as mean ± standard deviation. Differences between groups were compared by one- or two-way Analysis of Variance (ANOVA) (unless otherwise specified), and Duncan's methods as post-hoc test. Significant difference was indicated by $p<0.05$.

Results

Cell Culture and Identification of hUCMSC

After human umbilical cord tissue block was cultured for 5-7 days, new cells could be observed around most of the tissue block and diffused distribution. Most of them were spindle or long spindle-shaped fibroid cells with two protrusions, and a few of them were polygonal cells with multiple protrusions. The diopter of cell was well under inverted phase contrast microscope, the nucleus and nucleolus were obvious (Figure 1A-1B). At this time, the medium was changed. After it was changed every 3-4 d, the number of cells around the tissue block were significantly increased after 16-20 d culture, and > 90% cells

were fused. The cell morphology became long and thin, was similar with fibroblast-like cell with radial pattern or whirl distribution (Figure 1C). At this point, cells were to passage after digested by trypsin (1:2). The hUCMSCs after trypsin digestion were round, and adhered and stretched within 24 hours after passage. The hUCMSCs were still spindle-shaped or polygonal. After passage, the MSCs grew rapidly and the incubation period of proliferation was short. After grown for 3-4 days, more than 80-90% of the cells were fused. Cells were long spindle-shaped, radial pattern or paliform distribution (Figure 1D-1E). At this time, the cells needed to be passaged again. After cultured for 10-13 generations *in vitro*, the proliferation rate of the cell was slowed down and the cell was aged. Most of the cells showed large, flat polygon and lost their ability to proliferate and differentiate (Figure 1F). Flow cytometry results showed that human umbilical cord mesenchymal stem cells expressed cluster of differentiation (CD) 73, CD90, and CD105, but not CD14, CD34, CD45, and Human Leukocyte Antigen DR-1 (HLA-DR) (Figure 1G-1N). CD105 and CD73 are related markers of mesenchymal stem cells, CD90 is a mesenchymal related antigen, CD14 is a surface marker of monocyte macrophage, and CD34 and CD45 are positive markers of hematopoietic stem cells. The results of this study indicated that the surface-specific antigen markers of mesenchymal stem cells from human umbilical cord were consistent with mesenchymal stem cells from other tissues such as bone marrow and umbilical cord blood without expression of HLA-DR, indicating that its immunogenicity was weak.

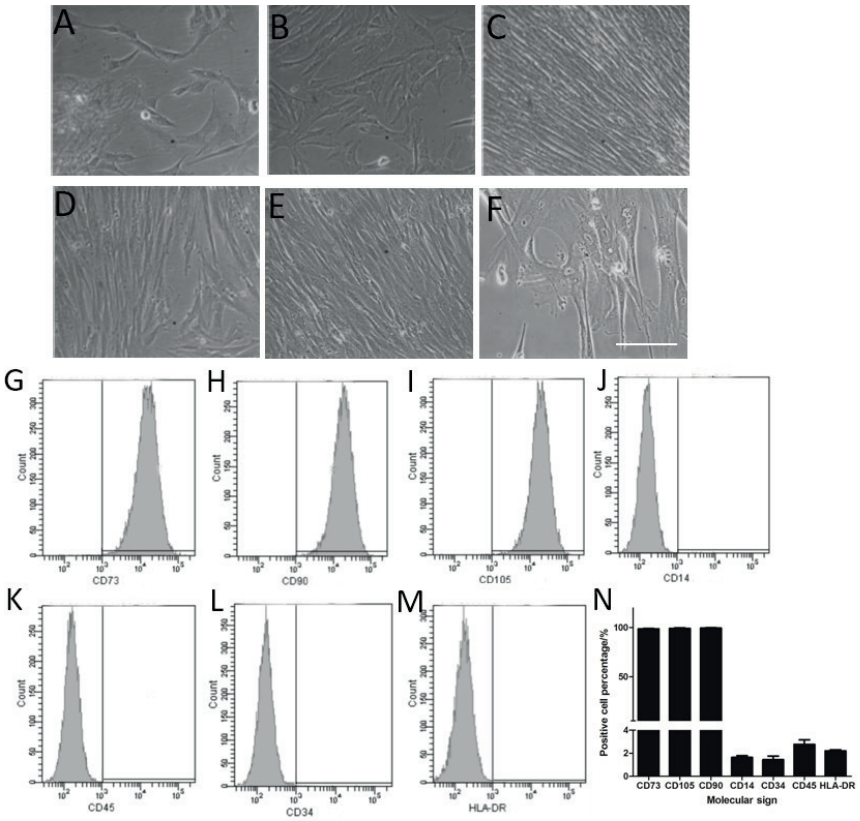

Figure 1. Cell culture and identification of hUCMSC. ***A,*** Spindle-shaped and polygonal cells could be seen on 6 d of primary generation; ***B,*** Spindle-shaped and polygonal cells could be observed on 11 d of primary generation; ***C,*** A large number of spindle-shaped fibroblast-like cells could be seen on 16 d of primary generation; ***D***, Spindle-shaped and polygonal cells could be seen on 2 d of 2nd generation; ***E,*** Spindle cells with radial pattern distribution on 3 d of 3rd generation; ***F,*** 11th generation on the 8th day, the cells were enlarged, flat and polygonal, and cell proliferation became slower on 8 d of 11th generation. ***G-N,*** Flow cytometry was used to detect that CD73 (A), CD90 (B), and CD105 (C) were expressed on the surface of hUCMSC cells, and CD14 (D), CD34 (E), CD45 (F), HLA-DR (G) were not expressed. Bar=50 μm.

Motor Function Recovery Promoted by hUCMSC Transplantation in SCI Model Mice

To study the therapeutic repair effects of hUCMSC transplantation on spinal cord in SCI model mice, LFB staining was used to detect the degradation of myelin at 2 weeks, 6 weeks, and 10 weeks in mice after SCI. The results showed: on 2 weeks after SCI, there was no significant difference in the amount of undegraded myelin between mice that hUCMSC were performed on the 10th day after SCI and mice in blank control group; The myelin content in mice treated with hUCMSC transplantation was increased significantly at the 6th and 10th weeks of SCI, and both were higher than that of the control group at the same time ($p<0.05$) (Figure 2A). At the same time, the results of NF neurofilament immunofluorescence staining of injured spinal cord in mice also showed that the neuronal content of spinal cord at the 6th and 10th week after SCI injury in the SCI model mice treated with hUCMSC was significantly higher than in the control group at the same time ($p<0.05$) (Figure 2B). Given that hUCMSC transplantation therapy

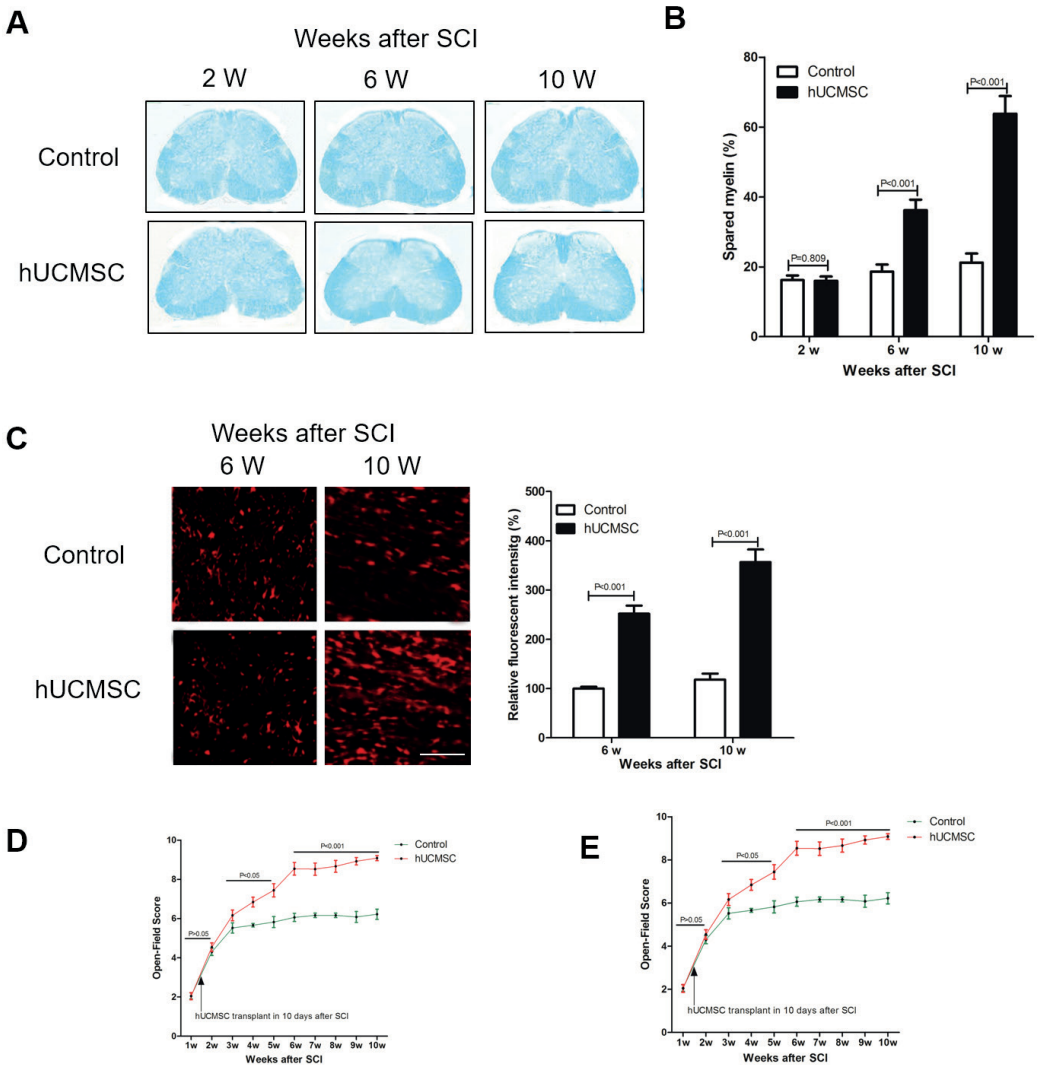

Figure 2. Motor function recovery promoted by hUCMSC transplantation in SCI model mice. A+B: LBP staining was used to detect bone marrow phospholipid degradation in spinal cord injury at 2 weeks (N=5), 6 weeks (N=5) and 10 weeks (N=5) after SCI. Scale bar =100 μl; C: NF neurofilament immunofluorescence staining was performed at 6 weeks (N=5) and 10 weeks (N=5) after SCI to detect neuronal cell survival in the spinal cord injury area. Scale bar = 50 μl; D: Recovery of hindlimb function in mice at different times after SCI injury; E: Retention time of mice in Rota-Rod device at different times of SCI injury. Data were expressed as (mean ± standard deviation). Bar=50 μm.

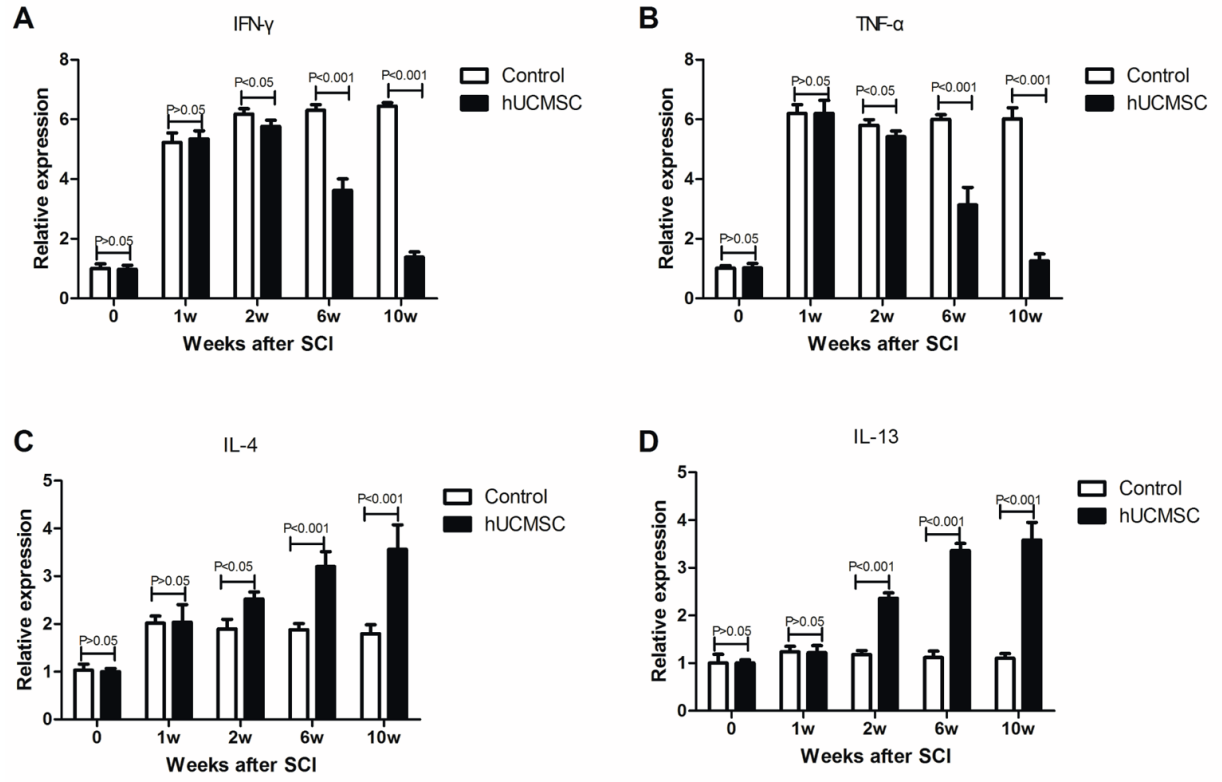

Figure 3. Th1 and Th2 cytokines in SCI model mice changed by hUCMSC transplantation. RT-qPCR was used to detect the expression of IFN-γ (A), TNF-α (B), IL-4 (C) and IL-13 (D) mRNA in rat spinal cord injury area at different time after SCI. 0 referred to normal mice without SCI.

could effectively improve histopathology of SCI model mice, we used behavioral test to investigate whether these histopathological improvements were also accompanied by better motor function recovery. The results showed that 4 d after transplantation therapy (that was 2 weeks after SCI injury), as for the mice treated with hUCMSC, open-field test scores or residence time on the Rota-Rod test device were significantly higher than mice in the control group at the same time ($p<$ 0.05) (Figure 2D and 2E), showing that hUCMSC transplantation could effectively improve motor function in SCI mice.

Th1 and Th2 Cytokines in SCI Model Mice Changed by hUCMSC Transplantation

Inflammatory cytokines played key roles in the pathological process of SCI. The expression of inflammatory cytokines were high in early stage after SCI and then decreased, and could be maintained to higher levels to mediate the corresponding inflammatory cells that invaded the injured spinal cord, producing an inflammatory response to aggravate the secondary damage to the spinal cord[23,24]. Our previous studies[21] had indicated that mRNAs of interferon (IFN)-γ, Tumor Necrosis Factor (TNF)-α, IL-4, and IL-13 in the spinal cord area after SCI in mice rapidly were increased in the early stage of SCI, and then maintained at higher levels for 2 weeks after injury. This study found that IFN-γ and TNF-α showed decreasing trend after hUCMSC transplantation on the 10th day after SCI in mice (Figure 3A-B), and IL-4 and IL-13 showed upward trend (Figure 3C-D). After 2 weeks of SCI injury, the expression levels of IFN-γ (Figure 3A) and TNF-α (Figure 3B) were significantly lower in mice treated with hUCMSC transplantation than mice in control group ($p<0.05$), while IL-4 (Figure 3C) and IL-13 (Figure 3D) were significantly higher than mice in control group ($p<0.05$). hUCMSC transplantation in the treatment of SCI mice could change the inflammatory response in the injured spinal cord of SCI mice by altering the production of Th1 and Th2 cytokines. It was further suggested that hUCMSC transplantation in the treatment of SCI mice may activate the differentiation of macrophages after SCI.

Activation of M2 Macrophages Promoted by hUCMSC Transplantation in Injured Site of SCI Mice

In the above study, we found that hUCMSC transplantation could reduce the inflammatory response by altering the production of Th1 and Th2 cytokines in the injured spinal cord. In order to determine whether hUCMSC transplantation therapy could influence the polarization of M1 and M2 macrophages after SCI in mice. The polarization of M1 and M2 macrophages in the injured spinal cord of mice were detected by flow cytometry and immunohistochemistry. After 4 days (i.e., after 2 weeks of SCI) of hUCMSC transplantation in SCI model mice, iNOS$^+$ macrophage population in the spinal cord area was significantly changed to the Arginase+macrophage population (Figure 4A-B), which further indicated that compared with mice in control group, the number of iNOS$^+$ macrophages in mice treated with hUCMSC transplantation were significantly reduced, and Arginase+ macrophages were significantly elevated (Figure 4C-D). These data clearly showed that for SCI injured mice, hUCMSC transplantation therapy could promote the preferential differentiation of macrophages into M2 macrophages and reduce the M1 macrophage population, resulting in unbalanced state of M1/M2 macrophage ratio.

Expression of IL-7 Reduced by hUCMSC Transplantation in Injured Site

Our previous study[21] had confirmed that blocking of IL-7 signaling pathway after SCI in mice could influence the release of pro-inflammatory factors by regulating the phenotype and function of macrophages, thereby reducing the inflammatory response in the injured site of spinal cord, helping to promote functional recovery after SCI in mice and achieve therapeutic effect on injured spinal cord. Then, whether the hUCMSC transplantation in treatment of SCI mice could change the macrophage phenotype by altering the expression of IL-7. Western blotting (Figure 5A) and RT-qPCR (Figure 5B) were used to detect the expression of IL-7 in the injured site of spinal cord at different times after SCI in mice among different groups. The results showed that after SCI, IL-7 was increased in the injured site of spinal cord, reached its peak after 2 weeks of SCI, and then decreased gradually, but it remained at a higher level (compared with uninjured spinal cord); mice were treated by hUCMSC transplantation

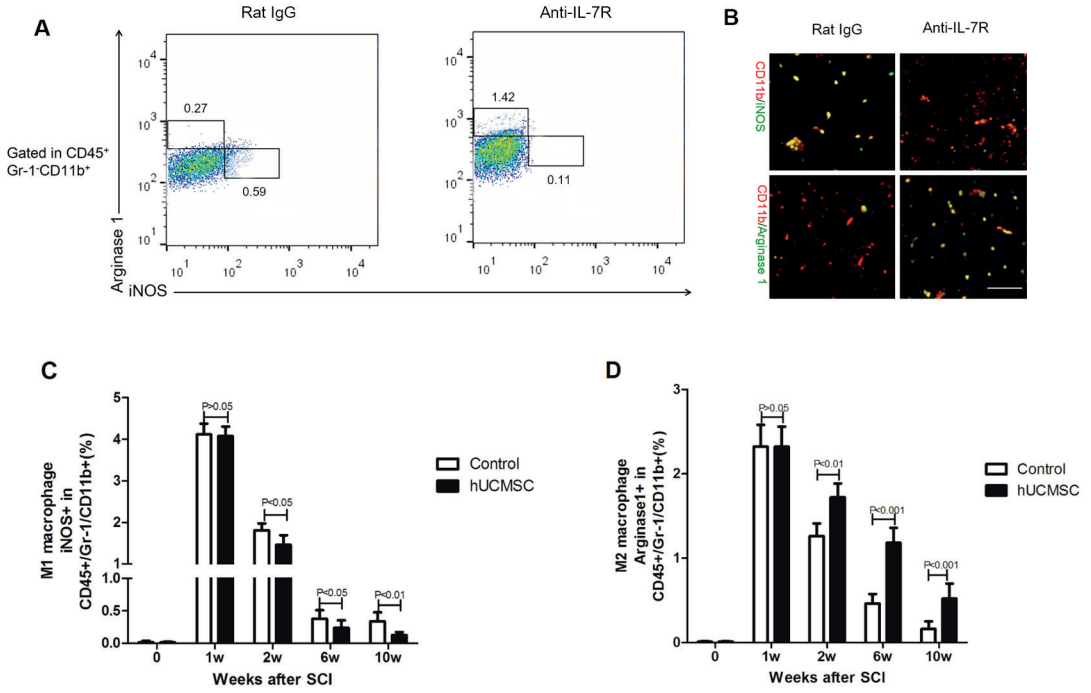

Figure 4. Activation of M2 macrophages promoted by hUCMSC transplantation. A+B: Marker expression of M1 (iNOS) or M2 (Arginase1) macrophages detected by flow cytometry ***(A)*** and immunofluorescence ***(B)*** after 6 weeks of SCI, scale bar = 50 μm; ***C+D,*** Percent statistics of M1 or M2 macrophages in injured site of spinal cord at different times after SCI. 0 referred to normal mice without SCI. Bar=50 μm.

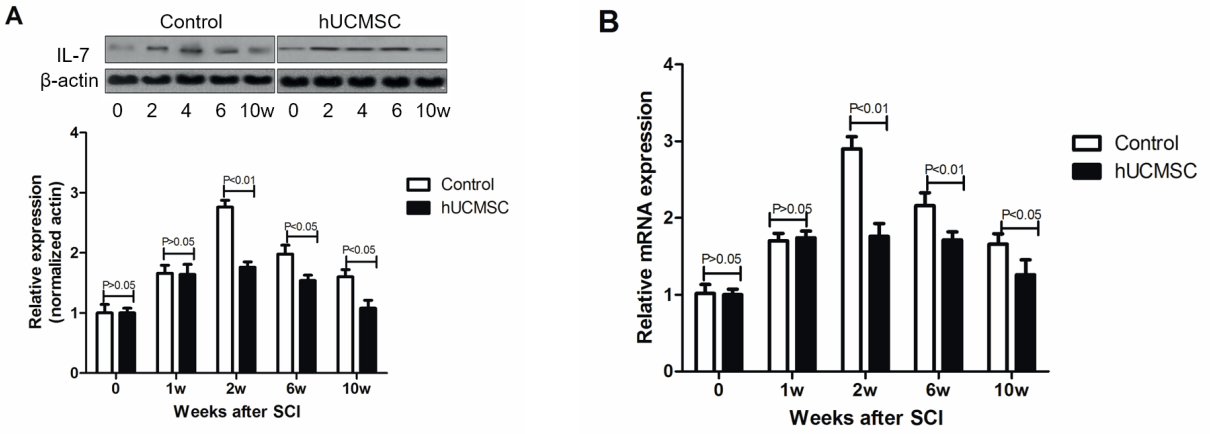

Figure 5. Expression of IL-7 reduced by hUCMSC transplantation in injured site of spinal cord. Immunoblotting (A) and RT-qPCR were used to detect the expression level of IL-7 in the injured site of mouse spinal cord among different groups at different times after SCI. 0 referred to normal mice without SCI.

on the 10th day after SCI injury, the expression level of IL-7 in injured site of spinal cord after 2 weeks of SCI was gradually decreased, and was significantly lower than mice in control group at 2, 6, and 10 weeks after SCI. These researches indicated that hUCMSC transplantation in the treatment of SCI mice could continue to reduce the expression of IL-7 in the injured site of spinal cord. Combined with the above analyses and our previous works, hUCMSC transplantation in the treatment of SCI mice could reduce expression of IL-7 in the injured site, promote the polarization of M2 macrophages, weaken the inflammatory response in the injured site, and promote the repair of injured site and improve motor function.

Discussion

The rise of stem cells and tissue engineering technology has brought new dawn to the study and application of nerve injury repair. One of the most promising treatments was stem cell transplantation that was used to treat SCI[25]. Different types of stem cells had been evaluated, including human umbilical cord blood mesenchymal stem cells[16,17]. The umbilical cord was a cord-like tissue that connects the fetus to the placenta. The surface of the umbilical cord was covered by amniotic membrane with grayish-white, and there were one umbilical vein and two umbilical arteries in the center of cord. Collagen-like embryonic connective tissue from the extraembryonic mesoderm with rich water content was around blood vessel, and called as Wharton jelly. From the perspective of embryonic development, the umbilical cord is the place where stem cells form and pass. Many totipotent or pluripotent stem cells, such as primordial germ cells, hematopoietic stem cells, needed to reach the embryo or fetus from the yolk sac via the Wharton jelly to form the corresponding tissue cells[26]. Therefore, the umbilical cord was likely to be rich in primitive cells and became tissue that could obtain abundant pluripotent stem cells. The human umbilical cord blood mesenchymal stem cell was a new type of seed cells for the treatment of SCI, and had received widespread attention.

In the animal experiments, Weiss et al[27] transplanted HUC-MSCs without any chemical inducer into Parkinson's rat model. It was found that the limb function of the transplanted rats was significantly improved and damaged neurons were obtained at a certain degree of recovery, so hUCMSC showed broad application prospects in nerve repair. At present, the way of HUC-MSCs transplantation for the treatment of SCI mainly focused on intramedullary direct local transplantation. This study used this method to perform hUCMSC transplantation on the 10th day after SCI in mice. Compared with the control mice, the hUCMSC transplantation in treatment of SCI mice not only promoted the survival of myelin and nerve cells in the injured site of spinal cord, but also significantly improved the motor function of SCI mice. In the previous study, human umbilical cord mesenchymal stem cells were transplanted into the spinal cord of rat model of completely

transected spine, and the transplanted cells could survive for a long period of time, which significantly restored the hind limb function of the spine transected rats[28]. The initial extraction was performed. After the initially extracted umbilical cord mesenchymal stem cells were transplanted into the moderately injured part of the injured site of spinal cord in rats, the transplanted cells were able to survive successfully, and they were able to fuse with the cells at the injury site, effectively accelerating axon regeneration[29]. However, so far, the best transplantation time for umbilical cord mesenchymal stem cells was still in dispute. As a result of our previous study, IL-7 expression in the injured site of spinal cord reached peak at 10th day after SCI (in dynamic fluctuation state 10 days after SCI), and subsequently increased to higher level and was kept at a high point. One of the objectives of this study was to investigate whether hUCMSC transplantation achieved therapeutic effects by modulating the expression of IL-7 in the injured site of spinal cord. Therefore, the hUCMSC transplantation time in this study was the 10th day after SCI.

Since umbilical cord mesenchymal stem cells did not express HLA-DR, the immunogenicity of UCMSC was weak. This was the basis for transplantation of hUCMSC to treat SCI model mice without producing immune rejection. Medicetty et al[30] and Weiss et al[27] transplanted porcine UCMSC into the brain of rats, and found that there was no rejection reaction. Cells could survive and proliferate at least 8 weeks in the host. No any immunosuppressive agents were used in transplantation of human UCMSC for treatment of Parkinson's disease in rats, and there was no immune rejection[31]. Because UCMSC had low immunogenicity and immunoregulatory function[32], and the results were obtained from the previous studies, hUCMSC transplantation therapy had wider range of applications. However, it was similar to other stem cell transplantation therapies for SCI, the specific mechanism of action of hUCMSC transplantation in the treatment of SCI was not yet completely clear. Inflammation is a pathophysiological response of body to injury factors *in vivo* and *in vitro*. In intact body, any local inflammatory process can affect the whole body and result in corresponding systemic response. In the early stages of inflammation, inflammatory cells infiltrated and produced inflammatory mediators and cytokines to eliminate foreign matter and kill bacteria[33]. When the inflammatory response was out of control (i.e. the cascade of cytoinflammatory factors occurred), the inflammatory response was amplified, and the corresponding anti-inflammatory response system was activated, resulting in damage to the normal tissue cells that had long been in inflammatory environment. Therefore, moderate inflammatory response was beneficial to the body and helped to eliminate infection, foreign matter, and aging cells, but excessive or sustained inflammatory responses cause varying degrees of tissue damage[34,35]. Macrophages are innate immune cells that exist in all tissues of the body, can phagocytize and kill intracellular parasites, bacteria, tumor cells, and cells that are aging and dead, and can also exert their immune defenses and immune self-stability, immune surveillance and other functions[36]. There were a variety of activation forms, and they were activated into different phenotype macrophages depending on the microenvironment[37]. Recent works[38,39] have shown that activated macrophages after SCI could be polarized into two cell subsets (M1 and M2), namely the proinflammatory "classically activated" macrophages and anti-inflammatory "alternatively activated" macrophages. In general, M1 macrophages had the characteristics such as damaging nerves and inhibiting nerve regeneration in secondary inflammatory reactions. Due to these characteristics, M1 macrophages benefited formation of glial scar that inhibited axon regeneration, and produced a large number of inflammatory mediators, inducing that micro-environment of injured site was not conducive to nerve regeneration[40]. Activated M1 macrophages produced high levels of inflammatory cytokines (IL-12, IL-23, IL-1β, and TNF-α) and cytotoxicity factor (iNOS)[41], and had strong phagocytosis and antigen-presenting capacity[42], so they were often described as pro-inflammatory cells[43]. Markers of M1 macrophages in secondary inflammatory response after SCI included iNOS, Nitrous Oxide (NOX), CD16/32 and CD86[44,45]. M2 macrophages could highly express IL-10 and Transforming Growth Factor (TGF)-β, reduce nuclear factor kappa B (NF-κB) activity, up-regulate Arg1 and down-regulate pro-inflammatory factors, so they were often described as anti-inflammatory cells[46]. In this study, it was found that hUCMSC transplantation not only reduced the expression of IL-7 in the injured site of spinal cord in SCI mice, but also promoted the activation of M2 macrophage and altered the production of Th1/Th2 cytokines. Our previous study[21] have showed that blocking of IL-7 signaling contributed to the polarization and migration of M2 macrophages, thereby af-

fecting the production of Th1 and Th2 cytokines and inhibiting the inflammatory response in the injured site of spinal cord to achieved therapeutic effects that prompted repair of injured site of spinal cord and recovery of motor function. Combined with this study, hUCMSC transplantation therapy could achieve the therapeutic effect of promoting repair of SCI and recovery of motor function by reducing the expression of IL-7 in the injured site of spinal cord.

Conclusions

We found that hUCMSC transplantation in the treatment of SCI mice could effectively improve the survival and regeneration of myelin and nerve cells in the injured site of spinal cord, and significantly improve the motor function of SCI mice. These gratifying therapeutic effects may be at least partially attributed to hUCMSC transplantation that could reduce the expression of IL-7 in the injured site of spinal cord, and then promote the activation of M2 macrophages and inhibit the inflammatory infiltration at the injured site.

Conflict of interest

The authors declare no conflicts of interest.

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