Effect of Bone Marrow Mesenchymal Stem Cell Transplant on Synovial Proliferation in Rats With Type II Collagen-Induced Arthritis

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Abstract

Objectives: To investigate the influence and mechanism of bone marrow mesenchymal stem cell transplant in the synovial proliferation of type II collagen-induced arthritis.

Materials and Methods: From the bone marrow of Sprague-Dawley rats, mesenchymal stem cells were isolated and expanded. Forty rats were randomly divided into 5 groups: normal control, early mesenchymal stem cell treatment, late mesenchymal stem cell treatment, early collagen-induced arthritis control, and late collagen-induced arthritis control. The mesenchymal stem cells and normal saline were injected through the tail vein, and the following parameters were observed: arthritis index, articular pathology changes, serum vascular endothelial growth factor level, tumor necrosis factor-α, and interleukin-17 levels as detected through stable enzyme-linked immunosorbent assay.

Results: The arthritis index and articular pathologic scores of the early and late treatment groups were lower compared with those of the control groups (P < .05). The arthritis index and articular pathologic scores of the late treatment group were lower than those of the early treatment group (P < .05). The levels of vascular endothelial growth factor, tumor necrosis factor-α, and interleukin-17 of the early and late treatment groups were significantly decreased compared with the collagen-induced arthritis control groups (P < .05), and these levels were positively correlated with the arthritis index and articular pathologic scores (P < .05).

Conclusions: The transplant of mesenchymal stem cells in rats with collagen-induced arthritis can inhibit the proliferation of synovium, which may be attributed to the reduced expression of vascular endothelial growth factor, tumor necrosis factor-α, and interleukin-17.

Key words: Bone mesenchymal stem cells, Transplant, Synovial proliferation

Introduction

Rheumatoid arthritis (RA) is a T-cell mediated systemic autoimmune disease, with its main symptoms including synovial proliferation and pannus. Angiogenesis, the formation of new blood vessels, is an earliest histopathologic finding in patients with RA that has been attributed to pannus development, induction of synovial inflammation, and destruction of osteochondral tissue. Therefore, the inhibition of synovial proliferation may be a therapeutic strategy for patients with RA.

Mesenchymal stem cells (MSCs) are a type of stem cell present in bone marrow. These stem cells do not express molecular major histocompatibility complex II or costimulatory molecules B7-1 and B7-2. Thus, they have a low level of immunogenicity. Cocultured MSCs with allogeneic peripheral blood mononuclear cells or allogeneic T cells do not cause proliferation of allogeneic T cells, indicating the feasibility of stem cell transplant. Several studies have indicated that MSCs produce beneficial effects on autoimmune disease. The immunosuppressive activity of MSCs in patients with RA has been studied in recent research. However, the role of MSCs in synovium proliferation is inconclusive. In the present study, we investigated the influence and probability of bone marrow MSC...
transplant in the synovial proliferation of type II collagen-induced arthritis (CIA).

Materials and Methods

Materials
Male Sprague-Dawley rats, weighing 180 ± 20 grams, were purchased from Shanghai Slaccas Experimental Animal Center (No. 180 SCXK, Hu 2007-0005). This study strictly followed recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. The animal use protocol was reviewed and approved by the Institutional Animal Care and Use Committee of the No. 3 People’s Hospital Affiliated to Shanghai Jiao Tong University School of Medicine in China.

Isolation, culture, and differentiation of stem cells
Bone marrow from Sprague-Dawley rats was flushed out of the femurs and tibias using low-glucose Dulbecco’s modified eagle’s medium. The MSCs were washed and plated at a concentration of 1 × 10⁷ cells/cm² in low-glucose Dulbecco’s modified eagle’s medium supplemented with 10% fetal calf serum, 100 U/mL penicillin, and 100 U/mL streptomycin. The MSCs were cultured in a humidified atmosphere with 5% CO₂ at a temperature of 37°C. The medium was initially replaced after 2 days, and thereafter once every 3 days. Adherent cells were collected with 0.25% trypsin/0.02% ethylenediaminetetraacetic acid and replaced when the cellular colonies reached a confluence of 80% to 90%. Third-generation MSCs were identified and used in the experiment.

Mesenchymal stem cells were recovered through incubation with 0.25% trypsin/0.02% ethylenediaminetetraacetic acid, counted at a concentration of 1 × 10⁶ cells/mL in phosphate buffered saline, stained with CD29/CD45 coupled to fluorescein isothiocyanate for 30 minutes, and washed with phosphate buffered saline 2 or 3 times. Afterward, flow cytometric analysis was performed.

The following steps were conducted after all the cells reached confluence: (1) The culture medium was cleared, and 2 mL adipogenic A was added; (2) adipogenic A was cleared and 2 mL adipogenic B was added after 3 days; (3) adipogenic B was cleared and 2 mL adipogenic A was added after 1 day. Adipogenic A and adipogenic B were circulated 3 to 5 times, and then counted in adipogenic B at 7 days. Thereafter, counts were performed once every 3 days. Adipogenic differentiation was assayed by Oil Red O.

Rat model and grouping
The concentration of the cattle collagen type II emulsion was 1 mg/mL. As much as 5 mg cattle collagen type II was dissolved in 2.5 mL 0.01 M glacial acetic acid at pH 3.2 before the test was conducted at 4°C. Then, 2.5 mL complete Freund’s adjuvant was added and emulsification was repeated. The rats were initially injected with 0.5 mL immune complex at the base of the rats root and at the back of the rats at the first immunity, and then were intraperitoneally injected with 0.5 mL immune complex after 14 days.

Forty rats were randomly divided into 5 groups: a normal control group, an early MSC treatment group (ie, MSCs were transplanted when the rats were induced for the first time), a late MSC treatment group (ie, MSCs were transplanted when the rats were induced for the second time), an early CIA control group (ie, normal saline was injected when the rats were induced for the first time), and a late CIA control group (ie, normal saline was injected when the rats were induced for the second time). The MSCs and normal saline (1 × 10⁷ cell/kg) were injected through the tail vein.

Clinical assessment of arthritis
The development of CIA was inspected every other day, and inflammation of the 4 paws was graded from 0 to 4 as follows: grade 0, paw with no swelling and focal redness; grade 1, paw with swelling of finger joints; grade 2, paw with mild swelling of ankle or wrist joints; grade 3, severe inflammation of the entire paw; and grade 4, paw with deformity or ankylosis. Each paw was graded and the 4 scores were totaled to obtain the maximum possible score of 16 per mouse. Rats were killed on day 60. The knees and ankles were fixed (with calcium removed), wrapped with paraffin wax, sliced, and stained with hematoxylin-eosin. The slides were evaluated histologically by 2 independent observers. The gradation of arthritis was scored on a 4-point scale: 0, normal; 1, mild; 2, moderate; and 3, severe. This score was assigned according to the intensity of mononuclear cell infiltration, pannus formation, lining layer hyperplasia, and subchondral bone destruction process, as described by Takayanagi and associates. The 4 index scores of rat arthritis were totaled for arthritis assessment.
Vascular endothelial growth factor, tumor necrosis factor-α, and interleukin 17
Blood was collected from eye socket of the rats, and serum vascular endothelial growth factor (VEGF), tumor necrosis factor (TNF)-α, and interleukin (IL)-17 were detected using the stable enzyme-linked immunosorbent assay.

Statistical analyses
Data are expressed as means ± SD. The ANOVA and the t test were used to determine statistically significant differences between groups. Statistical significance was defined as $P < .05$. Statistical analyses were conducted with SPSS software (SPSS: An IBM Company, version 17.0, IBM Corporation, Armonk, New York, USA) and organized with Excel 2010 (Microsoft Corporation, Redmond, WA, USA).

Results
Stem cell culture and identification
A litter of anchorage-dependent cells was found on the third day. However, MSCs could not be identified only from the cell morphology on the third day. When the cells were in the log phase on days 3 to 6, clones appeared more visible, but they were not of uniform size. After the cells reached 80% to 90% confluence, they were subcultured on 1:2 to 3 generations. Cell surface antigen was detected via flow cytometry. The CD29+/CD45- surface markers and MSCs reached 98.4% confluence after the subculture of 3 generations (Figure 1).

Most MSCs were differentiated for fat cells. They were found positive with Oil Red O dye after adipogenesis was induced (Figure 2A) and with Alizarin Red O dye after osteogenesis was induced (Figure 2B).

Arthritis index
Statistically significant differences in arthritis index (AI) ($P < .05$) were found between the normal control group and all experimental groups. Statistically significant differences in AI existed between the early MSC treatment group ($5.75 ± 0.46$) and early CIA control group ($9 ± 1.31$) ($P < .05$), as well as between the late MSC treatment group ($7.0 ± 0.76$) and late CIA

Figure 1. Fluorescein Isothiocyanate-Labeled Mesenchymal Stem Cells
(A) CD29 fluorescein isothiocyanate-labeled mesenchymal stem cells, with a positive expression rate of 98.4%.
(B) CD45 fluorescein isothiocyanate-labeled mesenchymal stem cells, with a positive expression rate of 3.2%.

Figure 2. Mesenchymal Stem Cells
(A) Mesenchymal stem cells, with Oil Red O dye at ×100 magnification after adipogenesis was induced.
(B) Mesenchymal stem cells, with Alizarin Red O dye at ×100 magnification, after osteogenesis was induced.
control group (8.75 ± 0.89) (P < .05). The AI was lower and swelling was lighter in the early MSC treatment group than in the late MSC treatment group (P = .004, Figure 3).

**Figure 3. Comparison of Arthritis Index in Groups of Rats Used in Study**

<table>
<thead>
<tr>
<th>Group</th>
<th>AI ± SD</th>
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<tbody>
<tr>
<td>Early CIA control</td>
<td>8.3 ± 1.4</td>
</tr>
<tr>
<td>Early MSCs treatment</td>
<td>3.5 ± 0.5</td>
</tr>
<tr>
<td>Late CIA control</td>
<td>7.0 ± 1.1</td>
</tr>
<tr>
<td>Late MSCs treatment</td>
<td>2.0 ± 0.0</td>
</tr>
<tr>
<td>Normal</td>
<td>7.0 ± 1.1</td>
</tr>
</tbody>
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Comparison of arthritis index in groups of rats used in study—normal control, early mesenchymal stem cell treatment, late mesenchymal stem cells treatment, early collagen-induced arthritis control, and late collagen-induced arthritis control.

**Detection of joint pathologic condition**

In the normal control group, the synovial cells arranged in 1 or 2 layers; hyperplasia, pannus, and inflammatory cell infiltration were absent; and the surface was smooth (Figure 4A). The synovial layer was thickened and extended to the articular cavity, with severe inflammatory cell infiltration and angiogenesis (Figure 4B). The early MSC treatment group (Figure 4C) and late MSC treatment group (Figure 4D) showed few synovial layer lesions and little inflammatory cell infiltration or cartilage damage.

The pathologic score of the early MSC treatment group (2.0 ± 0.0) was lower than that of the early CIA control group (8.3 ± 1.4), and the pathologic score of the late MSC treatment group (3.5 ± 0.5) was lower than that of the CIA control group (7.0 ± 1.1) (P < .05). Moreover, the pathologic score of the early MSC treatment group (2.0 ± 0.0) was lower than that of the late MSC treatment group (3.5 ± 0.5) (P < .05).

**Vascular endothelial growth factor, tumor necrosis factor-α, and interleukin 17**

The concentrations of VEGF, TNF-α, and IL-17 in the CIA control groups were higher than those in the normal control group. The VEGF, TNF-α, and IL-17 concentrations, AI, and joint pathologic score in the early and late MSC treatment groups were lower than those of the corresponding control groups (P < .05). In addition, the VEGF, TNF-α, and IL-17 concentrations,
AI, and joint pathologic score of the early MSC treatment group were lower than those of the late MSC treatment group ($P < .05$).

The correlation analysis showed that the concentrations of VEGF, TNF-α, and IL-17 had statistically significant positive correlations with AI and joint pathologic score (AI: $r=0.928$, $r=0.88$, $r=0.72$; pathologic score: $r=0.94$, $r=0.95$, $r=0.62$; $P < .01$ for all comparisons) (Table 1).

<table>
<thead>
<tr>
<th>Name</th>
<th>VEGF</th>
<th>TNF-α</th>
<th>IL-17</th>
</tr>
</thead>
<tbody>
<tr>
<td>AI</td>
<td>0.928*</td>
<td>0.880*</td>
<td>0.720*</td>
</tr>
<tr>
<td>Joint pathologic score</td>
<td>0.940*</td>
<td>0.950*</td>
<td>0.620*</td>
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</table>

* $P < .01$

**Table 1. Correlations of VEGF, TNF-α, IL-17 With AI and Joint Pathologic Score**

Some angiogenic-promoting factors are highly expressed in the synovial organization of RA, including VEGF, transforming growth factor-β, and epidermal growth factor. The most important factor in synovitis is VEGF, which has a greater-than-normal concentration in the serum and synovia of patients and animals with RA. In our experiment, VEGF expression was significantly increased in rats with arthritis, and its concentration showed a positive correlation with AI and pathologic score.

Tumor necrosis factor-α is an inflammatory cytokine that plays a key role in response to stress, and it can induce other inflammatory cytokines and break the balance within the cytokine network. Tumor necrosis factor-α also induces synovioblast and microvascular growth, and then induced the formation of the RA characteristic pannus.

Peripheral blood mononuclear cells and macrophages with synovium secrete TNF-α in patients with RA, increasing inflammatory markers and painful swelling of the joint. In the synovium of patients with RA, TNF-α functions in RA as follows:

1. TNF-α agglomerates and activates inflammatory cells. The activated vascular endothelial cell then induces the expression of adhesion molecules, extending the inflammation to the joint and pannus.
2. Tumor necrosis factor-α is induced to make matrix metalloproteinases from synovial cartilage cells and fibroblast, thereby degrading the articular cartilage and collagenous fiber.
3. Tumor necrosis factor-α helps synovial macrophages to further differentiate into osteoclasts. In the present study, TNF-α in the treatment groups was lower than that in the control groups and showed significant positive correlation with AI and pathologic score.

Interleukin 17 stimulates secretion of cytokines and promoting pannus formation in the synovium. Zhang and associates reported that IL-17 can mediate synovial cell hyperplasia by stimulating the expression of the proteins Cyr61 and integrin. Like TNF-α, IL-17 is induced to make matrix metalloproteinases from synovial cartilage cells and fibroblast, degrading articular cartilage and collagenous fiber. In our study, levels of IL-17 in the treatment groups were lower than those of the control groups and showed significant positive correlation with AI and pathologic score. The concentrations of VEGF, TNF-α, and IL-17 were higher in the CIA control groups than in the normal control group, showing a

**Discussion**

In terms of symptoms and disease processes in the present animal model study, CIA is the same as RA. Mesenchymal stem cells have a high capacity for self-renewal while maintaining multipotency, and they are capable of differentiating into multiple cell types, including adipocytes, cardiomyocytes, chondrocytes, and osteocytes. Mesenchymal stem cells have been differentiated on the basis of morphologic characteristics, induction of differentiation, and surface antigens. In our study, we found that MSCs with 98.4% CD29+/CD45- after subculture of 3 generations could induce adipogenesis or osteogenesis (Figures 1 and 2).

The rats exhibited no changes in their skin, fur, and internal bodies after MSCs were transplanted. However, joint swelling, confined activity, AI, pathologic score, and other parameters were improved. This result indicates that MSCs can be effective in CIA treatment, especially in the early stages of CIA.

Rheumatoid arthritis is a T-cell–mediated systemic autoimmune disease with main symptoms that include synovial proliferation and pannus. Angiogenesis, the formation of new blood vessels, is one of the earliest histopathologic findings in RA and is necessary for pannus development, induction of synovial inflammation, and destruction of osteochondral tissue. Most hyperplasia synovial cells are fibroblastlike synoviocytes. Serum vascular endothelial growth factor, TNF-α, and IL-17 are important factors in RA synovitis.
significant positive correlation with AI and pathologic score. The transplant of MSCs inhibited the secretion of VEGF, TNF-α, and IL-17, as well as the management of joint synovial inflammation. Therefore, MSC transplant can inhibit CIA synovial proliferation.

Various, complex factors induce synovial proliferation. The effects of RA factors other than VEGF, TNF-α, and IL-17 after MSC transplant require further studies to clarify. The expression of VEGF, TNF-α, and IL-17 also requires deeper discussion. The transplant of MSCs in patients with CIA may inhibit proliferation of the synovium and reduce expression of VEGF, TNF-α, and IL-17.

References