Transplanting of Mesenchymal Stem Cells May Affect Proliferation and Function of CD4⁺T Cells in Experimental Autoimmune Encephalomyelitis

Jingsong Zhu, Junfeng Zhang, Qianru Li, Ying Du, Baoping Qiao, Xiang Hu

Abstract

Objectives: To research the effects of transplanting mesenchymal stem cells on the development of experimental autoimmune encephalomyelitis and the mechanism behind it.

Materials and Methods: An experimental autoimmune encephalomyelitis animal model was induced by injection of the MOG peptide, and mesenchymal stem cell injection was done 20 and 22 days after experimental autoimmune encephalomyelitis induction. Clinical scores were recorded daily to evaluate developing experimental autoimmune encephalomyelitis. The frequency of CD4⁺CD25⁺Foxp3⁺T cells in the spleen, the thymus, and the lymph nodes were analyzed by flow cytometry, and Foxp3, TGF-β1, and IL-10 mRNA were detected by reverse-transcription-polymerase chain reaction.

Results: Transplant of mesenchymal stem cells on experimental autoimmune encephalomyelitis mice led to a decreased clinical score, an up-regulation of CD4⁺CD25⁺Foxp3⁺T cells, Foxp3, TGF-β1, and IL-10 mRNA in the spleen, the lymph nodes, and the thymus as compared with experimental autoimmune encephalomyelitis mice.

Conclusions: Transplant of mesenchymal stem cells may prevent developing experimental autoimmune encephalomyelitis and might be an available method in therapy of multiple sclerosis. Mesenchymal stem cells transplant may affect proliferation and function of CD4⁺T cells in experimental autoimmune encephalomyelitis, and CD4⁺CD25⁺Foxp3⁺T cell, Foxp3, TGF-β1, and IL-10 may be involved in this process.

Key words: Mesenchymal stem cells, Experimental autoimmune encephalomyelitis, Foxp3, TGF-β1, IL-10

Introduction

Multiple sclerosis (MS) is a chronic, disabling disease of the central nervous system (CNS). It is characterized by chronic neuroinflammation with lymphocyte infiltration into the CNS, myelin loss, various degrees of axonal and oligodendrocyte disorders, and progressive neurologic dysfunction. Experimental autoimmune encephalomyelitis is an autoimmune disease model of MS that has been used to study the mechanism of MS pathogenesis and to test the effectiveness of potential therapeutic agents for treating MS. A great deal of study in immunopathogenesis confirmed that the autoantigen of myelin protein in patients was recognized by CD4⁺T cells and an induced immune response. The immune cells were abnormally activated and transmigrated into the CNS, then interacted with cells expressing self-myelin protein antigens in the CNS, and finally led to demyelination of the CNS via a series of inflammatory factors. The demyelination of the CNS was concerned with the overactivity of effector T cells, infiltration of inflammatory cells in the CNS, increased production of proinflammatory cytokines, and inflammatory molecules. Cytokines such as...
interferon (IFN)-γ, tumor necrosis factor (TNF)-α, IL-2, and IL-17 produced by Th1 and Th17 cells could aggravate the illness, even IFN-γ could induce demyelination and neuronal damage. Th2 cells and CD4+CD25+ regulatory T cells (Treg) could inhibit the autoimmune reaction and limit disease progression by producing cytokines such as IL-10, TGF-β1, and IL-4. The number and immune suppression function of Tregs have been studied and have been reduced in experimental autoimmune encephalomyelitis (EAE) mice; when input functional Tregs into the mice, the pathogenetic condition was improved.

Mesenchymal stem cells (MSCs) are multipotential nonhematopoietic cells, and exist widely in many tissues such as bone marrow and liver. Mesenchymal stem cells are multipotent, self-renewing cells, and have the potential of multidirectional differentiation. They can differentiate into various tissues of mesodermal origin such as osteocytes, chondrocytes, adipocytes. The cells can be obtained easily, have great expansive potential in vitro, and have weak antigenicity, so MSCs have a good clinical application prospect. Mesenchymal stem cells also have been found to possess significant immunoregulatory activities. Mesenchymal stem cells could suppress the proliferations of T cells, B cells and NK cells, induce production of Tregs, and inhibit the maturation and function of antigen-presenting cell. Various studies have shown that MSCs have a potential application prospect in the treatment of autoimmune diseases, but the mechanisms mediating such effects are still not clear; that is the purpose of this study.

Materials and Methods

Animals
Female C57BL/6J mice, 6- to 8-week-old, and male BALB/c mice, 6- to 8-week-old, were purchased from the experimental animal institute, Chinese academy of medical science. Mice were housed in the department of microbiology and immunology, Zhengzhou University, China. Mice were maintained in a pathogen-free environment. All work was performed in accordance with the guidelines for animal use and care. All mice used in this study were bred and maintained in accordance with the guidelines set forth by The Care and Use of Laboratory Animals published by the China National Institute of Health.

Antigen
Mouse MOG35-55 peptide (MEVGWYRSPFSRV VHLYRNGK) was synthesized by Shanghai Sangon Biological Engineering Technology Co. Ltd (Song Jiang, Shanghai, China), and purity was confirmed by high-performance liquid chromatography.

Isolation and culture of mice mesenchymal stem cells
Bone marrow cells of C57BL/6J and BALB/c mice were flushed from mice femurs and tibias with phosphate buffer saline (PBS). Red blood cells were lysed, and the remaining bone marrow cells plated in MSC expansion media and were incubated at a concentration of 10^6/mL at 37°C in a 5% CO₂ atmosphere. After 72 hours, nonadherent cells were removed. When the plate was 80% to 90% confluent, adherent cells were trypsinized and split. Adherent MSCs were selected and expanded for 10 to 15 days. Cells were then trypsinized and used for tail vein injections into host mice (10^6 cells injected per mouse).

Mesenchymal stem cell expansion media: high-glucose Dulbecco’s modified eagle’s medium (Gibco, Grand Island, NY, USA) containing heat-inactivated 10% fetal bovine serum, 50 μg/mL gentamycin, 2 mM L-glutamine, 100 μM nonessential amino acids, 10 mM HEPES, and 55 μM 2-Mecaptoethanol.

Induction of experimental autoimmune encephalomyelitis and assessment of nervous function
Experimental autoimmune encephalomyelitis was induced in C57BL/6J mice by immunization with an emulsion containing 200 μg of purified MOG peptide in PBS and an equal volume of complete Freund’ adjuvant (CFA, Sigma-Aldrich, Steinheim, Germany) containing 800 μg of nonviable desiccated Mycobacterium tuberculosis H37RA (Difco Laboratories, Detroit, MI, USA). A final volume of 0.2 mL emulsion was injected subcutaneously at 4 sites over the flanks at day of induction (day 0) and at day 7. In addition, 300 ng of pertussis toxin (Sigma, St. Louis, MO, USA) in 0.2 mL PBS was injected intraperitoneally at day of induction and at day 2. After EAE induction, mice were scored daily for EAE clinical signs, according to the following scores: 0, no signs; 1, partial loss of tail tonicity; 2, tail paralysis.
and hind limb weakness; 3, hind limb paralysis; 4, hind limb paralysis and forelimb weakness or paralysis; 5, death due to EAE.

Transplant of mesenchymal stem cells
Forty C57BL/6J mice were divided equally into 4 groups: A, B, C and D. The mice in groups A, B, and C were used for induction of EAE. Twenty and 22 days after EAE induction, MSCs (10^6 MSCs in a volume of 200 μL) or the same volume of PBS was injected into each mouse tail vein. The differences were that MSCs from BALB/c mice were injected into mice in group A (allogenic transplant group), MSCs from C57BL/6J mice were injected into mice in group B (syngenic transplant group), and PBS was injected into mice in group C (EAE group). Group D mice were not treated because they were controls.

Flow cytometric analysis
Cells were isolated from the thymus, spleens, mesenteric, and popliteal lymph nodes of mice 40 days after MSC transplant. Single-cell suspensions were prepared by mechanical disruption, and red blood cells were removed from the samples with lysis liquid. Cells were washed 3 times in PBS using centrifugation, resuspended in PBS containing 1% bovine serum albumin (Sigma-Aldrich) and 0.1% sodium azide. For surface staining of CD4 and CD25, cells were incubated with fluorochrome-conjugated antibodies (FITC-conjugated anti-mouse CD4 mAb 0.125 μg/10^6 cells and APC-conjugated anti-mouse CD25 mAb 0.06 μg/10^6 cells, [eBioscience, San Diego, CA, USA]) to the indicated cell surface markers at the recommended dilution of isotype control antibodies for 30 minutes on ice. For intracellular staining of Foxp3, cells were fixed and permeabilized with Foxp3 staining buffer (eBioscience). Permeabilized cells stained with PE-conjugated anti-mouse Foxp3 mAbs (0.5 μg/10^6 cells [eBioscience]). Stained cells were analyzed subsequently using a FACSCalibur (Becton Dickinson, Franklin Lakes, NJ, USA).

Detected expression of Foxp3, TGF-β1, and IL-10 mRNA by real-time reverse-transcription-polymerase chain reaction
Total RNA was isolated respectively from 10^7 cell pellets of thymus, spleens, and lymph nodes using Trizol reagent (Invitrogen Corporation ) at 20, 40, and 60 days after MSC transplant. The first strand cDNA was subsequently synthesized using the RT Kit (Takara Bio, Otsu, Japan) according to the manufacture’s instructions. Expression of Foxp3, TGF-β1, and IL-10 mRNA was determined by real-time reverse-transcription-polymerase chain reaction using SYBR green I real-time PCR Kit (Takara Bio). Thermocycler conditions comprised an initial holding at 50°C for 2 minutes and a subsequent holding at 95°C for 10 minutes, which was followed by a 2-step PCR program at 95°C for 5 seconds and 60°C for 31 seconds for 40 cycles. Data were collected and quantitatively analyzed on an ABI Prism 7300 Sequence Detection System (Applied Biosystems, Foster City, CA, USA). Sequences of PCR primer pairs were as follows: Foxp3, forward 5’-agg aga aag cgg ata cca-3’ and reverse 5’-tgg gag gac tac cga ggc-3’; IL-10, forward 5’-tgg gag gac tac cga ggc-3’ and reverse 5’-gca gta cta agg-3’; TGF-β1, forward 5’-cct gct gtc ttc cca gag-3’ and reverse 5’-gcc aac gtc cca gag-3’. GAPDH primer pairs: forward 5’-gtt gtt gcc ttc ttc gat-3’ and reverse 5’-gaa gta ctc gga gtt-3’.

Statistical analyses
Results are expressed as means ± SD. Data were analyzed by an analysis of variance using SPSS software (SPSS: An IBM Company, version 13.0, IBM Corporation, Armonk, New York, USA). A value of P < .05 was considered as a significant difference.

Results
Characterization of mesenchymal stem cells after incubation and proliferation in vitro
The morphology of MSCs isolated from primary cells after 24 hour’s incubation were small and round, after 3 to 4 days, part of the cells attached to the culture plates and displayed a spindle-shape and a strong refraction. By days 7 to 10, more cells attached, and the size of the cells increased (Figure 1A). Two weeks after incubation, the numbers of MSCs increased, and part of the cells clustered (Figure 1B). Three weeks after incubation, most of the cells clustered, and 80% to 90% of the cells became overlapped and anastomotic (Figure 1C). Passage of cells was cultured continually, the number and growth velocity of the cells increased gradually. Cells from 3 to 4 passages were used for transplantsing MSCs.
Transplant of mesenchymal stem cells could improve the clinical score of experimental autoimmune encephalomyelitis mice

Mice were scored daily for EAE clinical signs. The results showed that in the EAE group, at 14 days after EAE induction, the animals began to show clinical signs: they developed quickly from tail paralysis (score 1), to hind limb paralysis (score 2), hind limb paralysis (score 3), and also part of the animals showed forelimb weakness (score 4) on day 5. In the allogenic transplant group and the syngenic transplant group, results showed that the clinical score increased from 14 to 20 days after MOG immunization and decreased after MSC transplant (20 days after immunization). The clinical score decreased to its lowest and maintained this level 20 days after MSC injection. The scores significantly decreased 20 days after MSC injection into the allogenic transplant group and the syngenic transplant group as compared with the EAE group (Figure 2; \( P < .01 \)).

Transplant of mesenchymal stem cells may affect levels of CD4⁺CD25⁺Foxp3⁺T cells in the spleen, the lymph nodes, and the thymus in experimental autoimmune encephalomyelitis mice.

Thymus, spleen, mesenteric, and popliteal lymph nodes were collected from mice at 20 days after MSC transplant. Single-cell suspensions were prepared and stained with fluorochrome-conjugated antibodies for analysis of CD4⁺CD25⁺Foxp3⁺T cells. Our results showed that a significantly lower frequency of splenic CD4⁺CD25⁺Foxp3⁺T cells was observed in the EAE group as compared with controls (\( P = .032 \)), the allogenic transplant group (\( P < .01 \)), and the syngenic transplant group (\( P = .018 \)). The frequency of CD4⁺CD25⁺Foxp3⁺T cells from lymph nodes in the EAE group was significantly lower than that of controls, the allogenic transplant group, and the syngenic transplant group (\( P < .01 \)). The frequency of thymic CD4⁺CD25⁺Foxp3⁺T cells in the EAE group also was significantly lower than that of controls, the allogenic transplant group, and the syngenic transplant group (Figures 3, 4, and 5; \( P = .025 \), \( P < .01 \), \( P = .036 \)).

Alteration of Foxp3, TGF-β1, and IL-10 mRNA by mesenchymal stem cells transplant in experimental autoimmune encephalomyelitis mice

Total RNA was isolated from \( 10^7 \) cell pellets of the thymus and spleens at 20, 40, and 60 days after MSC transplant. Expression of Foxp3, TGF-β1, and IL-10 was detected by real-time reverse-transcription-polymerase chain reaction. The results show stable expression of Foxp3, TGF-1, and IL-10 mRNA were displayed in the spleen, the lymph nodes, and the thymus of controls. Low expression of Foxp3 mRNA were showed in the spleen, the lymph nodes, and the thymus of the EAE group 20 days after MSC transplant, then decreased gradually and became lowest 60 days after transplant. Significant
differences were shown 60 days after transplant in the spleen, the lymph nodes, and the thymus of the EAE group compared with controls (Figure 6; P < .01). Low expressions of Foxp3 mRNA were shown at 20 days after MSC transplant in the spleen, lymph nodes, and thymus of the EAE group compared with controls (Figure 6; P < .01). Low expressions of Foxp3 mRNA were shown at 20 days after MSC transplant in the spleen.
the lymph nodes, and the thymus of the allogenic transplant group, then increased gradually to the normal level and had significant differences among 3 organs compared with the EAE group 60 days after transplant (Figure 6; \( P < .01 \)).

Similar results were displayed in the syngenic transplant group (Figure 6; \( P < .01 \) vs the EAE group in the spleen, the lymph nodes, and the thymus 60 days after MSC transplant). Lower expression of TGF-\( \beta \)1 mRNA was displayed in the spleen, the lymph nodes, and the thymus in the EAE group, the allogenic transplant group, and the syngenic transplant group 20 days after MSC transplant. In the EAE group, the level of TGF-\( \beta \)1 mRNA decreased gradually and reached its lowest 60 days after MSC transplant (Figure 7; \( P < .01 \) vs controls). Levels of TGF-\( \beta \)1 mRNA in the allogenic transplant group and the syngenic transplant group increased gradually to the normal level and had significant differences compared with the EAE group 60 days after transplant (Figure 7; \( P < .01 \) vs the EAE group in the 3 organs). In Figure 8, we found that similar altering tendencies of IL-10 were shown in the 4 groups in the spleen, the lymph nodes, and the thymus, and significant differences were displayed in the allogenic transplant group and syngenic transplant group as compared with the EAE group (\( P < .01 \) vs the EAE group in the 3 organs).

**Discussion**

In this study, MOG peptide was injected subcutaneously into mice to induce EAE in an animal model, which closely mirrors the hallmarks of MS pathology and is used clinically to evaluate the development of EAE. The results showed that a subcutaneous injection of MOG peptide could induce an increased clinical score in association with a decreased frequency of CD4\(^+\)CD25\(^+\)Foxp3\(^+\)T cells, and decreased expression of Foxp3, TGF-\( \beta \)1, and

**Figure 7. Detection of TGF-\( \beta \)1 mRNA by RT-PCR**

**Detection of Splenic Foxp3 mRNA in Different Groups**

**Detection of Splenic Foxp3 mRNA in Lymph Nodes of Different Groups**

**Detection of Splenic Foxp3 mRNA in Different Groups**

**Detection of Splenic IL-10 mRNA in Different Groups**

**Detection of Splenic IL-10 mRNA in Different Groups**

**Detection of Splenic IL-10 mRNA in Different Groups**

Total RNA was isolated from 10\(^7\) cell pellets of spleens, lymph nodes, and thymuses at 20, 40m and 60 days after MSC transplant in different groups. Expression of TGF-\( \beta \)1 mRNA was determined by RT-PCR. Data are means ± SD (n=10 per condition).

**Abbreviations:** EAE, experimental autoimmune encephalomyelitis; MSC, mesenchymal stem cells; RT-PCR, reverse-transcription-polymerase chain reaction
IL-10 mRNA in the spleen, the lymph nodes, and the thymus. Transplant of MSCs (either allogenic or syngenic) 20 and 22 days after MOG immunization on EAE mice led to a decreased clinical score, an up-regulation of CD4+CD25+Foxp3+T cell, Foxp3, TGF-β1, and IL-10 mRNA in the spleen, the lymph nodes, and the thymus. These results suggest that transplanting of MSCs could prevent developing EAE and might be an available method in the therapy of MS. CD4+CD25+Foxp3+T cell, Foxp3, TGF-β1, and IL-10 may be involved in this process.

The MSCs have received a great deal of attention in recent years mainly owing to their immunoregulatory function. However, the underlying mechanism that MSCs may affect a wide array of immunologic functions remains largely unknown. In this study, we revealed the mechanism via researching the influence that MSC transplanting has on the EAE animal model. We recorded the clinical score every day in the experiments. The results showed that the clinical score of the EAE group increased 14 days after MOG injection. This was consistent with the EAE clinical course, and suggests that EAE animal models were successful. Mesenchymal stem cell transplants were performed 20 and 22 days after MOG immunization. The clinical scores of both the allogenic transplant group and the syngenic transplant group were decreased and reached their lowest 20 days after MSC transplant (significant difference as compared with the EAE group) and persisted at this level until the mice were killed (60 days after the MSC injection). It has been reported that MSCs penetrate into the inflamed spinal cord and persist there up to 3 months. Histopathologic evaluation of brains and spinal cords of MSC-treated EAE mice reveals a reduction in the total number of infiltrates and 85% of the great majorities of axons have been found intact at 42 days to 80 days after the MSC treatment. These results suggest that MSC transplant could ameliorate the course of EAE intensively, and the effect could exist for a long time in the target tissues.

The ability of CD4+CD25+ regulatory T cells to actively suppress activation and expansion of self-reactive T cells is critical for protecting from autoimmunity. It has been reported that CD4+CD25+Foxp3+ T cells play a critical role in the immunosuppressive activity of MSCs, and this activity is related to the percentage of CD4+CD25+ Tregs. Foxp3 is considered an important marker for regulatory T cells and CD4+CD25+Foxp3+ T cells are preferentially regarded as regulatory T cells. So, in this study, we analyzed the frequency of CD4+CD25+Foxp3+ T cells in mice with or without MSC transplant. The results displayed that several CD4+CD25+Foxp3+ T cells were significantly decreased in the EAE group compared with controls. Our results are consistent with a previous study. However, the frequencies of CD4+CD25+Foxp3+ T cells, both in the allogenic transplant group and the syngenic transplant group, were increased after MSC transplants. These results suggest that MSC transplant could up-regulate expression of CD4+CD25+Foxp3+ T cells. Combining the results of the clinical scores, we found that while the clinical scores were decreased after MSC transplant, expressions of CD4+CD25+Foxp3+ T cells were increased. This indicates that up-regulation of CD4+CD25+Foxp3+ T cells might play a certain role in amelioration of the EAE clinical course after MSC transplant.

Some studies have shown that MSCs can protect allogeneic immune responses and have immunosuppression via production of TGF-β1 and IL-10. TGF-β1 was the first molecule to be described as a mediator of the immunosuppressive properties of MSC. Interleukin-10 has been shown to play a critical role in the beneficial effect of bone MSCs in sepsis. Foxp3 has been identified as a key regulatory gene for developing and functioning of Treg, and may be implicated as a regulatory program for developing regulatory T cells. Foxp3 was involved in regulating immunosuppression mediated by Tregs by inducing production of TGF-β and IL-10.

So in this study, we recovered the spleen, the thymus, and lymph nodes of mice with or without MSC injection and detected Foxp3, TGF-β1, and IL-10 mRNA in the 3 organs to research the immunoregulatory mechanisms mediated by MSC transplant. The results show that expression of Foxp3 mRNA in the 3 organs were decreased in the EAE group compared with controls. This is consistent with previous results. However, expression of Foxp3 mRNA in the allogenic transplant group and syngenic transplant group increased after MSC transplants compared with the EAE group. This suggests that MSC transplants could induce expression of Foxp3 mRNA.

Expression of TGF-β1 mRNA also was up-regulated in the allogenic transplant group and the syngenic transplant group after MSC transplants.
compared with the EAE group; these results suggest that MSC transplants could alter expression of TGF-β1 mRNA. Expression of IL-10 mRNA also was increased in the allogenic transplant group and the syngenic transplant group after MSC transplants. Alterations of IL-10 mRNA were induced by MSC transplant. The increased expressions of Foxp3, TGF-β1, and IL-10 mRNA in the spleen, the thymus, and the lymph nodes of mice after MSC transplant suggest that MSCs could induce expression of Foxp3, TGF-β1, IL-10, and the immunosuppressive activity of MSCs in EAE might be realized by production of Foxp3, TGF-β1, IL-10, and the 3 cytokines may be involved in the amelioration of the EAE clinical course by MSC transplant.

Our study showed that MSC transplant may prevent development of the EAE course and MSCs transplant might be an available means of therapy in MS. It is likely that up-regulation of CD4+CD25+Foxp3+ T cells, Foxp3, TGF-β1, and IL-10 mRNA contribute to this phenomenon, and that CD4+CD25+Foxp3+ T cells, Foxp3, TGF-β1, and IL-10 may play critical roles in the immunosuppressive activity of MSCs. However, many studies have concluded that bone marrow MSC-driven immunosuppression results from a shift in Th1/Th2 balance.33, 34 Interleukin-17 is a key inflammatory cytokine in autoimmune disease and may play an important role in the development of EAE.38 We do not know whether Th1 and Th17 cells participate in the immunoregulatory activity of MSCs, or whether cytokines (e.g., IFN-γ and IL-17 produced by Th1 and Th17 cells) are altered by MSCs. More studies are required to address these issues.

References

7. Kroenke MA, Segal BM. Th1 and Th1 responses directed against the immunizing epitope, as opposed to secondary epitopes, dominate the autoimmune repertoire during relapses of experimental autoimmune encephalomyelitis. J Neurosci Res. 2007;85(8):1685-1693.


