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FOXP3 Gene Expression in Multiple Sclerosis Patients Pre- and Post Mesenchymal Stem Cell Therapy

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ABSTRACT

Multiple Sclerosis (MS) is an inflammatory demyelinating and neurodegenerative disorder of the central nervous system (CNS), which mainly affects young adults. Activated T lymphocytes promote the neuro-inflammatory cascade of MS by secreting pro-inflammatory cytokines and play a significant role in its pathogenesis. T lymphocytes may trigger the inflammation, which in turn leads to axonal loss and neurodegeneration observed in the course of MS.

Currently, there is no cure for MS, however, one of the most promising neuroprotective research tools consists of the use of bone marrow derived mesenchymal stem cells (MSC). This method promotes immune system regulation and possibly induces neurological repair and re-myelination of the damaged axons. Recent studies have shown that MSC exert an immune regulatory function and induce T regulatory-cell proliferation, therefore, it may serve as a potentially useful treatment for immune-mediated diseases such as MS.

In this pilot study a group of MS patients underwent MSC therapy and we assayed the expression of an X-linked transcription factor, *FoxP3*, as a specific marker of T Regulatory cells in peripheral blood, prior to and after the treatment. Using q RT-PCR for measurement of expression of *FoxP3* by peripheral blood mononuclear cells, we found that in all subjects, except for one, the expression of *FoxP3* at 6 months after intrathecal injection of MSC was significantly higher than the levels prior to treatment.

Such significant enhanced expression of *FoxP3* associated with clinical stability. Findings from this pilot study further support the potential of bone marrow derived MSC for treatment of MS patients.

Key Words: Mesenchymal Stem Cells (MSC); Multiple Sclerosis (MS); Transcription Factor (FOXP3); T Regulatory Cells (Treg)

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INTRODUCTION

Multiple Sclerosis is an autoimmune disease characterized by demyelinating of nerve cells and inflammation in the central nervous system (CNS)

leading to damage of the myelin sheath.[1] Destruction of myelin, which is axon's insulator, can distort impulses through the brain and spinal cord, leading to the sensational and dynamic disorders in MS patients.[2] Both environmental factors (specific viral infections), and genetic susceptibility to MS and their interplay lead to inflammation in MS which is mediated by activation of T lymphocytes. Activated T cells can recognize and destroy the myelin sheath of neurons´ perhaps through the antigenic mimicry mechanism, which results in disability. Evidence for this hypothesis is supported by the presence of activated T-cells in MS lesions.[2,3] Current methods like immunomodulatory therapies (IFN-β, glatiramer acetate and mitoxantrone) for the treatment of MS are not fully effective, due to their insufficiency in controlling self-reactive lymphocytes and promoting re-myelinating and regenerating mechanisms, leading to cumulative disability and irreversible axonal/neuronal damage.[4,5] Thus, better approaches are required for immuneregulation of the constant anti-CNS inflammatory process. Using bone marrow transplantation in treating autoimmune diseases provided an opportunity to achieve this goal. Preclinical results suggest that bone marrow cells may provide a source of stem cells, potentially capable of migrating into inflamed CNS and differentiating into cells expressing neuronal and glial cell markers.[6] Among the stem cells, it seems Mesenchymal Stem Cells (MSC) can be the best candidate for treatment of MS due to their ability in regulating immune responses and possibly induction of neurological repair and re-myelinization mechanisms. Perhaps through recruitment and activation of T-regulatory lymphocytes (Treg) which suppress T-cell proliferation, MSC put forth an immune regulatory function.[7]

Based on the findings that MSC regulate immune system activity and promote neuro-regeneration in the mouse model of chronic EAE,[8] we hypothesized that the use of a similar therapeutic method in MS patients with progressive disease who have already failed all currently available immunomodulatory agents, leads to stability and improvement of their clinical status. One potential mechanism for such improvement is the effect(s) of MSC on recruitment and function of Treg lymphocytes.[7,9] MSC suppress T-cell proliferation *in vitro*,[9] in animal model[10] and in humans.[11] The discovery of FoxP3 as a specific marker of Treg lymphocytes led to an expansion of research in biological properties of these cells. Functional features of FoxP3 is associated with CD4+ regulatory T lymphocytes.[12-14] FoxP3 appears to be predominantly expressed by the CD4+CD25+regulatory T lymphocytes (Treg), and ectopic expression of FoxP3 in CD4+CD25+ T lymphocytes is sufficient to convert them into Treg lymphocytes with strong suppressor activity.[12,15-17]

More notably, targeted mutation of FoxP3 in hematopoietic stem cells is both necessary and sufficient to enable Treg development.[13] Thus, FoxP3 is regarded as a major regulator for the lineage differentiation and function of Treg lymphocytes.[18] Studies involving MS patients have revealed that the number of regulatory T cells, particularly those expressing FoxP3 may change during disease processes.[19]

To test the hypothesis that injection of autologous MSC to patients with MS alters the activity of Treg lymphocytes, we examined the expression of Foxp3 (as the marker of Treg cells) in the peripheral blood mononuclear cells of seven MS patients prior and following the intrathecal injection of MSC.

MATERIALS AND METHODS

Seven patients with relapsing-remitting MS participated in this open-labeled clinical trial.The study was approved by the Ethics Committee of Tehran University of Medical Sciences and all study patients provided signed informed consent. Each study patient had experienced more than two relapses during the year prior to entry to the clinical trial and all had failed treatment with immunomodulatory agents and corticosteroids. Forty days prior to the initiation of clinical trial, 50 mL of autologous bone marrow (BM) were obtained from each study patient. The BM mononuclear cells (MNC) were separated by ficoll density gradient method. MNC were seeded in culture flasks with MSC medium, consisting of dulbecco's modified eagles medium and fetal bovine serum. Flasks were incubated at 37°C in a humidified atmosphere containing 5% CO2 for MSC expansion, during culture process, when expanded cells reached the desired number; they were prepared to be injection to the patients. In order to ascertain that cells were not contaminated, bacteriological tests were performed on the samples for every passage and at the time of injection. Viability of the cells was assessed by methylene blue dye exclusion test just prior to

injection. Next, a mean volume of 10 mL containing at least 20X106 cells were injected intrathecally to the patients. They were assessed and examined following the procedure and were admitted for 24 hours before being discharged from the hospital. Study patients were followed for six months. Peripheral blood mononuclear cells were obtained from 7 MS patients who received MSC.

Samples were collected at four points in time: day 0 prior to injection of stem cells and months 1, 3 and 6 after the intrathecal injection of MSC. Total RNA was extracted from PBMCs by TRYZOL (SIGMA) reagents, according to the protocol (Invitrogen LifeTechnologies). Agarose gel electrophoresis was done to check the RNA integrity (Figure1). Then, 1µg of this RNA converted to CDNA by Fermentase reagents according to protocol (from CINNAGENE M-MuLV Reverse Transcriptase #EP0351).

FoxP3 mRNA levels were quantified by real-time PCR with the ABI/PRISM 7500 sequence detection system (PE Applied Biosystems, Foster City, CA, USA). Real time quantitative polymerase chain reaction (qRT-PCR) was performed using SYBER GREEN I Gene Expression Assay for FoxP3. Relative expression was determined by normalization to UBC (Ubiquitin C) as a housekeeping gene. Specific primers were designed as follow: FOXP3 primers: FW-GAGAAGCTGAGTGCCATGCA, RWAGGAGCCCTTGTCGGATGAT,and UBC primers: FWATTTGGGTCGCGGTTCTTG, RW-TGCCTTGACATTCTCGATGGT.

Primers were designed to span exons so as not to anneal to contaminating genomic DNA. Each PCR sample contained 0.3 µM primers in a final volume of 20 µl, and amplification was carried out via 10 min at 95˚C denaturation step followed by 40 cycles of 15 s at 95˚C and 40 s at 60˚C. Melting curves of cDNAs were obtained via 15 s at 95˚C, 1m at 60˚Cand 15 s at 95˚C and used to calibrate the threshold cycle to relative quantities of FOXP3and UBC cDNAs in each sample. All samples were run in triplicates.

Relative FOXP3 expression levels were calculated as [2ΔΔ Ct], where ΔΔCt = [ΔCt(sample) - ΔCt (calibrator)] and ΔCt = [Ct (sample) –Ct (housekeeping)]. Statistical analysis was done with non- parametric test, Wilcoxon and Freedman, using SPSS software.

RESULTS

Seven patients, 1 male and 6 females, participated in this pilot open-labeled clinical trial.The average age of study patients was 35.5 years (range of 30-50 years). The average duration of disease prior to participation in this clinical trial was 4.9 years. Analysis of obtained results from q RT-PCR on PBMCs revealed that, in all subjects except one,expression of the FOXP3 was significantly increased compared to the period prior to treatment of the patients with autologous MSC ($p<$ 0.005) (Figure 2). The mean expression of mRNA of FOXP3 as a specific marker of Treg lymphocytes increased from 1 (prior to injection of autologous MSC) to 7.08 (6 months after the injection) (Table 1).

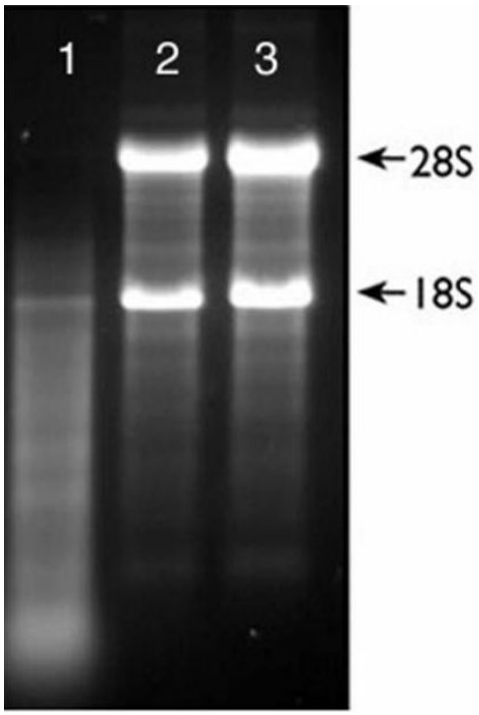

Figure 1. RNA integrity assesses. As shown in figure, one µg of total RNA was run on agarose gel and 28s and 18s bands were observed.

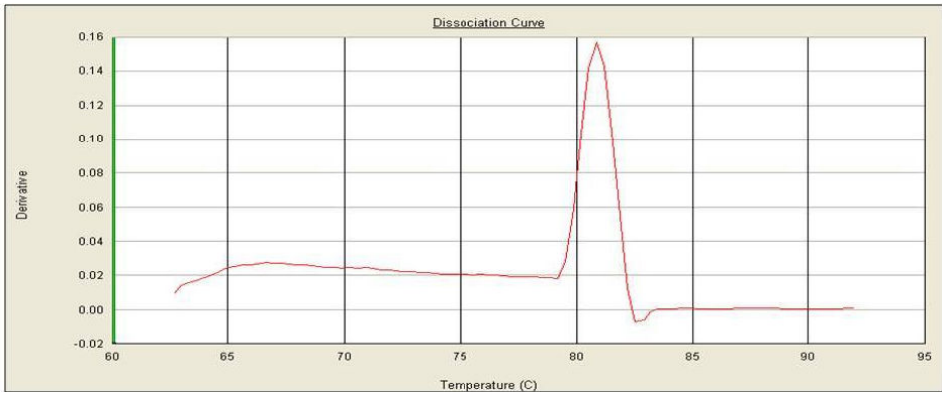

Figure 2. Melting curve for FOXP3 cDNA. This unique pick of the curve indicates that the amplified sequence was specific.

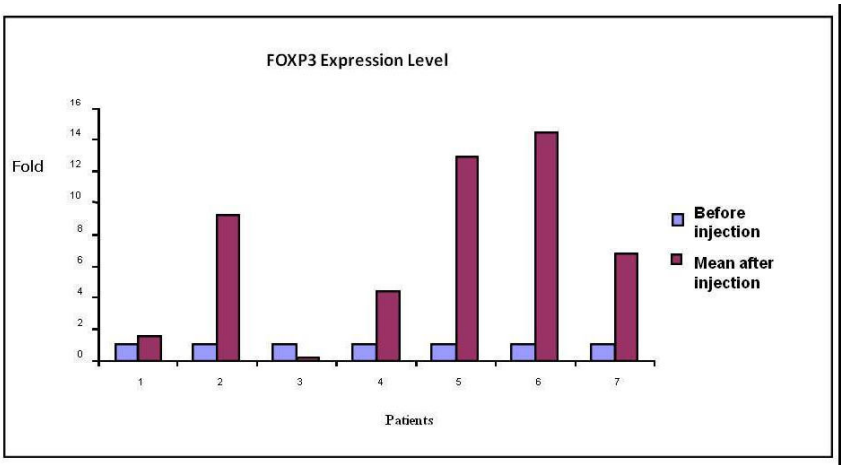

Figure 3. Quantitative analysis of *FoxP3* mRNA expressed in PBMCs driven from MS patients. Results in four stages indicate statistically significant differences in *FoxP3* expression level. Only the results of day 0 versus months 6 after the injection are shown.

Table1. Quantitative expression of FoxP3 mRNA expressed in PBMCs driven from MS patients

Name	Prior to injection of MSC	1 month after injection of MSC	3 months after injection of MSC	6 months after injection of MSC	Mean
Patient 1	**1**	**1.818778**	**2.81564**	**0.035466**	**1.556628**
Patient 2	**1**	**26.27146**	**1.010441**	**0.291241**	**9.191048**
Patient 3	**1**	**0.248369**	**0.273969**	**0.048902**	**0.190413**
Patient 4	**1**	**2.621844**	**0.190127**	**10.28043**	**4.364134**
Patient 5	**1**	**35.53369**	**1.901966**	**1.353789**	**12.92981**
Patient 6	**1**	**4.107489**	**6.183482**	**33.17239**	**14.48779**
Patient 7	**1**	**0.802617**	**1.161484**	**18.54656**	**6.836888**

DISCUSSION

The FoxP3 transcription factor is among the most definitive markers associated with regulatory T cells. CD4+/CD25+ regulatory lymphocytes are principally involved in maintenance of self-tolerance,[16] i.e., they suppress effector T-cell proliferation and cytokine production in a cytokine-independent way requiring cell-to-cell contact.[12] Aside from autoreactive T-cell suppression, CD4+/CD25+ cells also regulate immune responses against infectious pathogens, cancer, and allogeneic organ and stem cell grafts.[16] Only CD4+/CD25+ cells expressing the highest levels of CD25 (called CD25 brightcells) reported to be Treg. They are characterized by high expression of FoxP3 which is required for Treg development.[11] Regulatory T cells are competent to suppress harmful immune responses against self- or non-self-antigens. Recent findings that Foxp3 mutations lead to imperfect development of regulatory T lymphocytes and the appearance of a fatal autoimmune, inflammatory and allergic diseases suggest they exert positive role in tolerance and immune regulation.[20] Increasing data indicates that the level and duration of FOXP3 expression play a critical role in the development and function of Treg lymphocytes.[12] In some studies, an association between the number and the activity of Treg lymphocytes and disease status have been detected in autoimmune disease. Decrease in activity and the number of Treg lymphocyte appear to be present in the course of myasthenia gravis, autoimmune polyglandular syndrome type II, ulcerative colitis, and multiple sclerosis.[19,21-26] Understanding the response of FoxP3 post-translational modifications and the inter-relation of FoxP3 ensemble enzymatic components to physiological and pathological stimuli will introduce new pharmaceutical targets, providing potential therapeutics for modulation of Treg lymphocytes involved in transplantation, allergy, autoimmune diseases and cancer.

Impairment of Treg lymphocyte number and function may contribute to the development of MS.[26,27] MSC have been shown to increase the number of either CD4+CD25+lymphocytes or CD4+CD25+FoxP3+ lymphocytes in different in-vivo and in-vitro studies.[28]

Several mechanisms have been proposed for MSC immunomodulation: MSC can operate through production of soluble factors, through cell–cell contact or may operate as immature antigen presenting cells. Moreover, MSC can recruit, regulate, and maintain Treg lymphocyte function in co-culture experiments over time.[28] Induction of production of Treg lymphocytes by MSC in several studies [29] indicates that this may be a potential mechanism of action of MSC in ameliorating autoimmune diseases. In certain *in vitro* MSC and T-lymphocyte co-culture model, T lymphocytes demonstrated a regulatory phenotype[30-33] and recruitment of Treg lymphocytes has been hypothesized to participate in the down regulation of T lymphocytes' response by MSC.[34]

Consequently, advanced characterization of the MSC–Treg lymphocyte interaction and elucidation of the mechanisms of their interactions revealed that MSC were involved in Treg lymphocytes recruitment and regulation.[34] More recently, in a study on MS patients exhibiting advanced disability, it was shown that following injection of MSC the rate of T-reg lymphocyte markers were significantly increased.[35] However, to the best of our knowledge, no other studies has supported this hypothesis and therefore the role of MSC in restoring the number or function of Treg lymphocytes in the context of autoimmune disease remains merely speculative. In the present study, a supportive effect of MSC on the Treg lymphocytes was established by assay of mRNA expression of FoxP3 as a specific marker of these cells. Our study shows the mean of post-injection data was clearly significant compared to the data obtained from pre- injection stage in 6 of our 7 study subjects. Our data show, FoxP3 expression increases in MS patients after stem cell therapy. In our experiment, injection of MSC to MS patients, led to over expression of FoxP3 during six months period supporting the hypothesis of up-regulating of Treg lymphocytes by MSC. Therefore, increased expression of this gene may indicate an increase in the number or a boost in the activity of Treg lymphocytes in the majority of our cohort of MS patients.

CONCLUSION

The results of the present pilot study indicate that MSC may be effective in obtaining a sufficient number of Treg lymphocytes, particularly in the profusion of CD4+/CD25+ fraction for clinical purposes in MS patients because MSC increases the Treg number. These findings support former studies that employed MSC, through inducing Treg cells, can ameliorate the

symptoms of immune-mediated diseases such as MS.Therefore, it may be concluded that one of the effective mechanisms of MSC's function in treatment of autoimmunity is to induce and up regulate Treg in humans. As there are limited experiments on this issue, it seems that further studies using MSC at the earlier stages of the MS, when the irreversible neurodegenerative damages have not occurred, are necessary to promote certain degree of neuroregeneration in these patients. Furthermore, culturing of bone marrow-derived MSC, possibly supplemented with biological agents such as cytokines, can be beneficial to augment regeneration process in MS patients.

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