Evaluation of Cytokines in Multiple Sclerosis Patients Treated with Mesenchymal Stem Cells

Mohyeddin Bonab Mandana, a Mohajeri Maryam, b Sahraian Mohmmad Ali, c Yazdanifar Mahboubeh, a Aghsaei Aida, c Farazmand Ali, b and Nikbin Behrooz a

aResearch Center of Molecular Immunology, Tehran University of Medical Sciences, Tehran, Iran
bDepartment of Cell & Molecular Biology, School of Biology, Faculty of Science, University of Tehran, Tehran, Iran
Scina MS Research Center, Sina Hospital, Tehran University of Medical Sciences, Tehran, Iran

Received for publication August 17, 2012; accepted March 22, 2013 (ARCMED-D-12-00424).

Background and Aims. Mesenchymal stem cells (MSC) are currently strong candidates for stem cell therapy. Cytokines have a profound effect on the resultant immune responses. This study aims to evaluate variations in the cytokine profile of multiple sclerosis patients treated with autologous MSC.

Methods. Twenty five patients received one dose of intrathecal MSCs (mean number: 29.5 \( \times 10^6 \)). To measure the gene expression of FOXP3, IFN-\( \gamma \), TGF-\( \beta \), IL-4, IL-10, IL-6, and their serum proteins, samples were collected at five intervals: day 0 prior to injection and months 1, 3, 6, and 12 after MSC therapy. Gene expression was evaluated via real-time PCR and protein values were measured by ELISA.

Results. There were no statistically significant variations in gene expression and serum level of cytokines after a 1-year follow-up of MSC-treated MS patients. The only correlation found was an increase in IL-6 gene expression in patients with progressive disease.

Conclusion. Intrathecal injection of MSCs does not affect cytokine variation in peripheral blood. Because the condition of most of our patients either improved or stabilized after stem cell therapy (SCT), we speculate that the immunomodulatory or neuroregenerative effects of MSC are exerted locally in the central nervous system.

Key Words: Mesenchymal stem cells, Multiple sclerosis, IL-6 gene expression, Stem cell therapy.
cytokines that form the basis for their cell-mediated/pro-inflammatory (Th1, Th17), humoral immunity/anti-inflammatory (Th2), and suppression/regulation (Tr1, Treg CD4+CD25+) activity. Cytokines released from each of these subgroups have inter-regulatory effects on the other subgroup’s function. For instance, IFN-γ secreted from Th1 inhibits Th2 cells function and bilaterally IL-10 secreted from Treg cells blocks Th1 and Th2 cells (6,7). Dominance of each Th1, Th2 or Treg cytokines has a profound effect on resultant immune responses and may cause or change inflammation pattern (8).

An effective therapy for MS should be able to suppress activated cells against myelin sheath, which may be achieved through secretion of anti-inflammatory cytokines. To better understand the underlying mechanisms, we proposed to study cytokines and their gene expression in progressive MS patients who received MSCs. T regulatory lymphocyte (Treg) is one of the important factors in downregulation of activated immune responses in the periphery and inhibition of inflammation in injured organs (9). Among the most definitive markers associated with regulatory T cells is the FoxP3 transcription factor. CD4+/CD25+FoxP3+ regulatory lymphocytes are principally involved in maintaining self-tolerance (10). They suppress effector T-cell proliferation and cytokine production; therefore, they can play a positive role in tolerance and immune regulation (11,12). MSC has been shown to increase the number of either CD4+CD25+lymphocytes or CD4+CD25+FoxP3+ lymphocytes in different models and assays (13). MSC can recruit, regulate, and maintain Treg lymphocyte function in co-culture experiments over time (14). Induction of production of Treg lymphocytes by MSC indicates this may be a potential mechanism of MSC action in ameliorating autoimmune disease (15).

There may be a question if MSCs of MS patients have preserved their natural function. Mazzanti et al. studied MSCs and showed that there is no notable difference in the characteristics and functional capabilities of these cells derived from MS patients vs. healthy controls (16). Thus, MSCs can be used autologously for cell therapy in MS patients. Many in vitro studies have proved immunomodulatory effects of MSC by soluble mediators. The underlying mechanisms of the observed immunomodulatory effects have not been fully revealed in human-based studies. The purpose of the present study is to examine the effects of intrathecal injection of MSC on FOXP3+cells, IFN-γ, TGF-β, IL-4, IL-10 and IL-6 cytokines at gene expression and protein levels in the peripheral blood of progressive MS patients. Theoretically, it is not expected to see any changes in peripheral cytokine profile after intrathecal injection of MSCs. The fact applies as long as the blood brain barrier (BBB) is intact. Meanwhile, it is well known that the BBB may be disrupted during disease progression in MS patients (17,18). Therefore, we first tried to challenge the hypothesis in practice. Second, we investigated any correlation between clinical outcome of MSCs on MS patients and the peripheral cytokine profile.

Materials and Methods

In this study, 25 MS patients were recruited according to inclusion criteria previously described (19). Briefly, patients with clinically definite MS, with secondary progressive MS (SPMS) or progressive relapsing MS (PRMS) forms of the disease and aged between 18 and 50 years underwent MSC therapy. They had to be unresponsive to conventional MS treatments and had 1 score increase in Expanded Disability Status Scale (EDSS) or evidence of an inflammatory disease activity during 1 year prior to recruitment.

Details of MSC preparation have been reported in our previous study (19). In summary, 50 ml of bone marrow was aspirated from MS patients under sterile conditions. Mononuclear cells were isolated and cultured in MSC medium. MSCs were harvested after two or three passages. To confirm the stem cell characteristics, differentiation potential to adipocyte and osteocyte lineages was evaluated. Flow cytometry was also performed to determine surface expression of MSC markers: CD105, CD90, hematopoietic stem cell (HSC) markers: CD34, CD45, and endothelial cell marker CD31. The viability and sterility of cells were determined in every passage by trypan blue dye exclusion and bacteriological tests, respectively.

Blood samples were collected from these patients who had been treated by MSC to measure six gene expression and protein levels (except FOXP3, which has no secretory protein product). Gene expression was assayed by real-time PCR and protein values were measured by ELISA.

Patients were categorized into two groups according to their disease outcome after 1 year. In group 1, patients were in remission phase or somehow improved and group 2 patients were those who had increment in their EDSS or new lesions shown in their magnetic resonance imaging (MRI).

Sample Collection and RNA Extraction

Peripheral blood mononuclear cells (PBMCs) were obtained from 25 MS patients who received MSC. Samples were collected at five intervals: day 0 prior to injection of stem cells and months 1, 3, 6, and 12 after intrathecal injection of MSC. Total RNA was extracted from PBMCs by TRIZOL (Sigma, St. Louis, MO) reagents according to the protocol (Invitrogen Life Technologies, Carlsbad, CA). Agarose gel electrophoresis was done to verify RNA integrity. Next, 1 μg of this RNA was converted to cDNA by Fermentas reagents according to protocol (M-MuLV Reverse Transcriptase #EP0351).
Real-time PCR

FOXP3 and five other cytokine mRNA levels were quantified by real-time PCR with the ABI/PRISM 7500 sequence detection system (PE Applied Biosystems, Foster City, CA). Real-time quantitative polymerase chain reaction (qRT-PCR) was performed using SYBER GREEN Gene Expression Assay. Relative expression was determined by normalization to UBC (Ubiquitin C) and β-2 macroglobulin as housekeeping genes. Specific primers were designed as shown in Table 1. Primers were designed to span exons so as not to anneal to contaminating genomic DNA. Each PCR sample contained 0.3 μmol primers in a final volume of 20 μl, and amplification was carried out for 10 min at 95°C denaturation step followed by 40 cycles of 15 sec at 95°C and 40 sec at 60°C. Melting curves of cDNAs were obtained for 15 sec at 95°C, 1 min at 60°C and 15 sec at 95°C and used to calibrate the threshold cycle to relative quantities of FOXP3, UBC and β-2m cDNAs in each sample. Before running patient samples, all tests were set up with samples from healthy donors to achieve accurate results. All samples were run in triplicate. Relative gene expression levels were calculated as (2 Ct), where Ct = (Ct [sample] – Ct [calibrator]) and Ct = (Ct [sample] – Ct [housekeeping]).

ELISA Technique

Blood samples were centrifuged, and serum was then drained and frozen until processed. ELISA kits were used to assay cytokines IL-4, IL-6, IL-10, TGF-β and IFN-γ.

For the ELISA test, R&D system kit (Minneapolis, MN) was used and the test was performed according to the manufacturer’s instructions.

Statistical Methods

In order to normalize quantitative data, Ln transformation was used. Then, in order to compare our data at different times, before and after stem cell therapy, nonparametric Wilcoxon test, GEE model (generalized linear models) with correlation matrix exchangeable and linear regression formula were applied.

For studying the effects of gene expression and ELISA quantities on disease outcome, GEE test and correlation matrix were used. Because disease outcome is a binary variable, logistic regression formula was used.

Data analysis was performed by SPSS v19 software (Chicago, IL); p < 0.05 was considered significant.

Results

In this study 25 patients—23 SPMS and 2 PRMS—19 females and 6 males, participated with their consent and approval of the ethics committee. Average age of patients was 34.7 ± 7 (23–50) and EDSS range was between 5.5 and 7. The disease duration was >5 years in all cases. The mean dose of injected cells was 29.5 × 10⁶.

Comparison of overall gene expression and serum level of peripheral blood cytokines after intrathecal MSC therapy revealed no statistically significant variations compared to the results obtained before injection. Gene expression variations and protein levels of cytokines are depicted in Figures 1 and 2, respectively.

Gene expression and protein level variations of IL-4, IL-6, IL-10, IFN-γ and TGF-β were also evaluated based on disease outcome. IL-4 protein levels were undetectable in all patients. The only cytokine that showed a significant difference after treatment was IL-6 gene expression, which showed a remarkable increase (p = 0.03) in patients (group 2) who showed elevated EDSS and/or enhancement of plaque formation in MRI post-SCT (Table 2, Figure 3).

The clinical changes in our patients during 12 month post-MSC therapy were previously published (19). Briefly, MRI (3T) and EDSS scores improved or remained unchanged in 15 (68.18%) patients, whereas seven (31.81%) patients showed new T2 or gadolinium-enhanced lesions or increased EDSS. Three (13.63%) patients refused to undergo further follow-up after 6 months. The results of flow cytometry tests were positive for MSC markers (CD105, CD90) and negative for HSC markers (CD34, CD45) and endothelial cell marker CD31. The viability range of the cells was 96—100 and the results of bacteriology showed no growth for all samples. Osteocyte and adipocyte culture differentiation were positive for all patients.

Discussion

The fact that MSCs display significant anti-proliferative, anti-inflammatory and anti-apoptotic features has highlighted their therapeutic potentials. These features coupled with the reported plasticity and ability of MSCs to transdifferentiate into neural cells (20) and migration to the central
nervous system (CNS) (21) make them a unique candidate for various therapeutic applications in diseased and injured microenvironments.

It has been shown (22) that MSCs and their cytokine production (IL-6, TGF-β) can efficiently regulate FOXP3 and RORγT gene expression. TGF-β production by MSC induces FOXP3 gene expression, activating Tregs. Concurrently, TGF-β and IL-6 support RORγT expression, causing pro-inflammatory Th17 cell proliferation.

In one study, following intrathecal and i.v. injection of MSCs, the rate of Treg lymphocyte markers were significantly increased in MS patients exhibiting advanced disability (23).

IL-4 as an anti-inflammatory cytokine prevents Th1 cell activation and decreases IL-1 and TNF-α production in vitro. Amelioration of experimental autoimmune encephalomyelitis (EAE) has been related to stimulation of Th2 responses by IL-4. Reduced IFN-γ and increased IL-4 production/expression were found in the splenocyte culture supernatants and brain of EAE mice treated with minocycline and prednisone (24). It has been determined that serum concentration of IL-4 significantly elevates in the remission phase of MS patients (25).

IL-10 acts as an anti-inflammatory cytokine. It has been reported that IL-4- and IL-10-producing cells inhibit the Th1 cell response in EAE (26). Human studies have shown that IFN-β therapy leads to a significant increase in the production of IL-10 in MS patients, which results in pro-inflammatory cytokine reduction (27). In contrast, Martins et al. showed a significant increase in IL-4 and IL-10 in MS patients compared to healthy controls (28).

MSCs in the presence of low amounts of IFN-γ express higher levels of MHC-II on their surface and obtain antigen presenting cell (APC) phenotypes. In the presence of high amounts of IFN-γ, they express lower amounts of MHC-II, lose their APC function and acquire immunosuppressive

Figure 1. Variation in gene expression of IL-4, IL-10, TGF-β, IFN-γ, FOXP3 and IL-6 before (0) and after (months 1, 3, 6, 12) stem cell therapy.
properties, thus preventing DC maturation (29). Therefore, we propose that MSC in an inflammatory microenvironment (high IFN-γ) acts as an anti-inflammatory agent.

In our study, we did not find any significant changes in peripheral blood cytokines compared to values prior to treatment. As previously mentioned, there is a probability of disruption of the BBB in MS patients. As long as the BBB is intact, we do not expect any changes in peripheral cytokine levels post-intrathecal injection. Our study results may suggest that even if the BBB is disrupted during disease progression, it is not to the extent that affects the peripheral cytokine profile.

On the other hand, we observed improvement or stabilization in almost 70% of our patients after a 1-year follow-up. We were not able to correlate this improvement with cytokine variation profiles in the peripheral blood. This indicates a local effect (improved patient condition) while ruling out a global impact (cytokine profile change). How can we explain this phenomenon?

First, during CNS inflammation, most of the CNS leukocytes are T cells and the remaining 25% are B cells (30). One study showed that clinical progression of MS is associated with an increase of B cells in the CNS (31). In most chronic infections and autoimmune diseases, atypical lymphoid follicles are formed in target tissue. It appears that these follicles are responsible for local B cell and T cell priming (32). Presence of these atypical follicles in the meninges of secondary progressive multiple sclerosis (SPMS) patients was reported by Serafini (33). These organelles provide an appropriate microenvironment for B cell differentiation. Second, immunomodulatory effects of MSC, which lead to significant neuroprotection, have been clearly portrayed in EAE (15,34). MSC injection to animal models with cerebrospinal injury can lead to amelioration of disease and prolonged nerve cell survival. In experimental conditions, therapeutic effects of bone marrow-derived mesenchymal stem cells (BM-MSC) are mostly related to paracrine mechanisms through growth factor release, anti-apoptotic molecules, and anti-inflammatory cytokines. In particular, paracrine mechanisms can provide an appropriate environment for nerve regeneration, remyelination and cerebral flow improvement (35). Because the

![Figure 2. Variation in protein level of IL-6, IFN-γ, TGF-β and IL-10 before (0) and after (month 1, 3, 6, 12) stem cell therapy.](image-url)
Table 2. Gene expression (G) and protein level (P) variations correlate with disease outcome adjusted for time variable using GEE test with logistic regression formula

<table>
<thead>
<tr>
<th></th>
<th>G</th>
<th>P</th>
<th>G</th>
<th>P</th>
<th>G</th>
<th>P</th>
<th>G</th>
<th>P</th>
<th>G</th>
</tr>
</thead>
<tbody>
<tr>
<td>IL-4</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>IL-6</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>IL-10</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>IFN-γ</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>TGF-β</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>FOXP3</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*p 0.576 — 0.038 0.913 0.492 0.928 0.764 0.161 0.625 0.999 0.193

IL-4 protein levels were undetectable in all patients.

CNS of MS patients form its own reservoir of lymphoid follicles, we speculate that the autologous MSCs we inject into the subarachnoid space of our patients directly target and affect the immune cells differentiating within the CNS microenvironment. This correlates with the paracrine effects demonstrated by previous studies. This can also explain why peripheral effects are minimal and undetectable in our study.

As previously mentioned, our results showed increased levels of peripheral IL-6 gene expression in patients with disease progression (group 2). Compatible to our findings, several studies have demonstrated that IL-6 plays a role in progression, differentiation, and degeneration of neuronal cells in the peripheral nervous system (PNS) and the CNS (36). The harmful effects of IL-6 were first implicated in brain-related processes when its dysregulated expression was documented in several neurological disorders such as MS, Alzheimer’s disease and Parkinson’s disease (37). In EAE during disease relapse, the peak of the IL-6 gene expression was correlated with disease severity (38) and IL-6 deficient mice were resistant to EAE induction (39). Knowing the facts, increase in IL-6 levels in our study may simply be due to the natural course of the disease. In other words, MSC was not able to influence the course of the disease in this group of patients. Unfortunately, lack of a control group—the main limitation of the current study—does not help to clarify this issue.

In conclusion, to the best of our knowledge, most previous animal and clinical studies that observed significant cytokine variations in peripheral blood injected MSCs i.v. Our patients were treated intrathecally and most patients improved or stabilized. Unlike i.v.-injected MSC, we did not observe a significant variation in peripheral blood cytokines. Therefore, we believe that combined intrathecal and i.v. injection of MSC may boost the effectiveness and functionality of MSC both locally (in the CNS) and peripherally (in the blood). Further studies should be designed to treat patients with a combination of intrathecal and i.v. injections.

Acknowledgments

The authors appreciate Dr. Ahmadreza Shamshiri, faculty member of the Department of Epidemiology and Biostatistics, School of Health, Tehran University of Medical Sciences for assistance in statistical analysis. This work was supported by grant #6549-30-04-86 provided by Tehran University of Medical Sciences.

The authors declare no conflict of interest in regard to this manuscript.

References


Figure 3. IL-6 gene expression showed a remarkable increase (p = 0.03) in patients with disease progression (group 2). IL-6 gene expression correlation with disease outcome adjusted for time variable using GEE test with logistic regression formula.


26. Oh SJ, Chung DH. Invariant NKT cells producing IL-4 or IL-10, but not IFN-gamma, inhibit the Th1 response in experimental autoimmune encephalomyelitis, whereas none of these cells inhibits the Th17 response. J Immunol 2011;186:6815–6821.


