Neuroprotective Effects of Transplanted Mesenchymal Stromal Cells-derived Human Umbilical Cord Blood Neural Progenitor Cells in EAE

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ABSTRACT

Multiple Sclerosis (MS) is an autoimmune inflammatory demyelinating disease of the central nervous system. The aim of this study was to investigate the neuroprotective effects of transplanted human umbilical cord blood mesenchymal stromal cells (UCB-MSC) derived neural progenitor cell (MDNPC) in EAE, an experimental model of MS.

To initiate neuronal differentiation of UCB-MSCs, the pre-induction medium was removed and replaced with induction media containing retinoic acid, b FGF, h EGF, NGF, IBMX and ascorbic acid for one week.

The expression of neural genes was examined in comparison to control group by real-time PCR assay. Then, experimental autoimmune encephalitis (EAE) was induced using myelin oligodendrocyte glycoprotein (MOG, 35-55 peptides) in 24 C57BL/6 mice. After induction, the mice were divided in four groups (n=6) as follows: healthy, PBS, UCB-MSCs and MDNPC, respectively. At the end of the study, disease status in all the groups was analyzed using hematoxylin-eosin (H&E) staining of brain sections.

We found that UCB-MSCs exhibit neuronal differentiation potential in vitro and transplanted MDNPC lowered clinical score and reduced CNS leukocyte infiltration compared to untreated mice. Our results showed that MDNPC from UCB may be a proper candidate for regenerative therapy in MS and other neurodegenerative diseases.

Keywords: Differentiation; Experimental Autoimmune Encephalomyelitis; Mesenchymal stromal cells; Neural Stem Cells

INTRODUCTION

Multiple Sclerosis (MS) is an autoimmune inflammatory demyelinating disease of the central nervous system. The aim of this study was to investigate the neuroprotective effects of transplanted human umbilical cord blood mesenchymal stromal cells (UCB-MSC) derived neural progenitor cell (MDNPC) in EAE, an experimental model of MS.

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nervous system (CNS).1,2 When inflammation occurs in the brain, astrocytes are activated and involved in the process of reactive gliosis.3 However, etiology of MS and its pathogenesis needs further investigations.4

Experimental animal models are necessary prior to approving new medical treatments for regenerative medicine. EAE provides a suitable model for the study of pathogenesis and immune regulation of CD4+ T\textsubscript{eff}/T\textsubscript{reg}\textsuperscript{17}–mediated tissue damage, and is generally considered a relevant model for human immune-mediated demyelinating disease of multiple sclerosis.5

Current treatments have mostly reduced the disease relapse rate yet have not had a considerable impact on preventing the progression of MS.6 In recent years, ethical conflicts and legal restrictions have hindered the development and clinical application of ESCs and NSCs, which emphasizes evaluation of different stem cell sources in various cell-based therapies.7 Recent studies suggest that grafting MSCs into the brain could induce neuroprotective effects in EAE/MS therapy.8,9

Concerns about transplanting MSCs in the clinic demands further attention to MDNPC in EAE/MS therapy.10

Therefore, immunomodulatory and neuroprotective strategies are being studied as likely novel therapies for MS. MDNPC seem to be good candidates to control inflammation and myelin injury in EAE and MS due to production of different growth factors for promoting angiogenesis and mitosis of NSCs. These factors can also promote oligodendrogenesis and inhibit gliosis.11–15

It was shown for the first time that MDNPC from UCB can have a transient neuroprotective effect on EAE. UCB-MSCs are selected due to lower immunogenic potential, lack of graft versus host disease (GVHD), higher capacity for neuronal differentiation and non-invasive feature after delivery compared to other tissues.16

In our experiment, we designed a simple protocol for neuronal differentiation of umbilical cord blood–derived mesenchymal stromal cell (UCB-MSCs) cultured under appropriate conditions in vitro. Then, we investigated the neuroprotective effects of transplanted human umbilical cord blood MSC-derived neural progenitor cells (MDNPC) in EAE, an experimental model of MS. All of the aspects of our experiments that are presented are novel.

MATERIALS AND METHODS

Culture of UCB-MSCs

UCB-MSCs were isolated from human cord blood as previously described.17 In brief, the mononuclear cell (MNCs) fraction was separated by density centrifugation over a Ficoll-Hypaque gradient (Pharmacia-Amersham; d=1.077 g/ml). After two times washing in phosphate-buffered saline (PBS; Gibco, USA), the collected MNCs were re-suspended in high glucose-Dulbecco’s modified eagle medium (DMEM; Gibco, USA) supplemented with 10% fetal bovine serum (FBS; Gibco), L-glutamin (Gibco), 100 U/ml penicillin, and 100 mg/ml streptomycin (Gibco). MSCs were cultured in a humidified atmosphere of 95% air with 5% CO\textsubscript{2} at 37 °C. When the cells reached confluence, they were detached using 0.05% Trypsin-EDTA (Gibco) and plated in new 25 cm\textsuperscript{2} tissue-culture flasks in 10 ml cultured medium.

Flow Cytometry of UCB-MSCs

Flow cytometry was done as follows. After the second passage, the cells were trypsinized, washed two times with PBS and stained on ice by PE-CD14, CD29, CD44, CD45, CD73, CD105, CD106, CD271, HLA-DR, FITC-CD34 and Percp CY5.5-CD90 monoclonal antibodies according to manufacturer’s instructions (BD Biosciences, USA; except for monoclonal antibody against human CD34, which was from DAKO, Denmark). In the control group, PE-IgG1 and FITC-IgG1 antibodies were used. The stained cells were analyzed by flow cytometry (Partec Flomax, ver 2.4e).

Adipogenic and Osteogenic Differentiation

The differentiation of UCB-MSCs was assessed in cultures of the third passage. The cells were cultured in a medium which contained either osteogenic (L-glutamin (Sigma, USA), dexamethasone (Sigma, USA), β-glycerophosphate (Sigma, USA), and ascorbic acid A; Sigma, USA) or adipogenic (dexamethasone, insulin (Sigma, USA), indomethacin (Sigma, USA), and IBMX, Sigma, USA) materials. Two weeks later, osteogenic differentiation was assessed by the Alizarin-Red S 2% staining for mineralization capacity in osteocytes and adipogenic differentiation was assessed by the Oil Red-O staining to detect the presence of lipid vacuoles in adipocytes.
Neuronal Differentiation of UCB-MSCs

For in vitro neuronal differentiation, UCB-MSCs (20000 per well on a 24-well culture plate) at 70% confluence were cultured in an induction cocktail containing a basal medium (Gibco) supplemented with 2% B-27 (Gibco), L-glutamin, retinoic acid (AA, Sigma), basic fibroblast growth factor (bFGF, Sigma), epidermal growth factor (EGF, Sigma), nerve growth factor (NGF, R&D Systems, USA), 3-isobutylmethyl-xanthine (IBMX, Sigma), and ascorbic acid (AA, Sigma). The medium was changed two times per week. Neuronal differentiation was examined for morphology, and quantitative real-time PCR (qPCR) analyses in different time points were conducted for detecting neuronal differentiation.

Quantitative Real-Time PCR Analysis

Total RNA was isolated from neural undifferentiated and differentiated UCB-MSCs using an RNA isolation kit (Qiagen, USA), and reverse transcription was carried out using cDNA Kit (Invitrogen) according to the manufacturer's protocol. In order to confirm the expression of neural-specific genes, qPCR was performed in triplicate by SYBR green real-time master mix (Takara, Japan) in a Corbett 6,000 Rotor-gene (Corbett, Germany) followed by melting curve analysis to confirm PCR specificity. Relative expression was quantified using REST 2009 software. Nucleotide sequences and the amplicon size of the designed primers are listed in Table 1.

Induction of EAE

Twenty-four 8-10 week-old female C57BL/6 mice were purchased from Pasteur institute (Iran) and housed under standard humidity, 22-23°C temperature, and 12/12 h (7 am 7 pm) dark/light cycles in pathogen-free animal laboratory conditions. All experiments were carried out according to Iranian Blood Transfusion Organization (IBTO) guidelines for animal care. EAE induction was performed according to a previously published protocol.18

In brief, the mice were immunized subcutaneously with 200 µg of MOG 35–55 peptide (Alexis, Switzerland) in 100 µl PBS completely emulsified in 100 µl complete Freund's adjuvant (CFA; Sigma, USA). Immunization was followed by intra-peritoneal (IP) administration of 400 ng pertussis toxin (Sigma) in 200 µl PBS on day 0 and after 48 h.

Clinical Evaluations of EAE

Clinical scores were registered according to the standard protocol: 0=no clinical scores, 0.5= partially limp tail, 1=paralyzed tail, 2=loss in coordinated movement; hind limb paresis, 2.5=one hind limb paralyzed, 3=both hind limbs paralyzed, 3.5=hind limbs paralyzed; weakness in forelimbs, 4=forelimbs paralyzed, and 5=moribund with EAE. Body weight was also registered every 2 days.18

Treatment

The ability of UCB-MSCs & MDNPC to alleviate the clinical score of EAE was investigated by administration via intravenous route. In brief, within 14 days of post-EAE inductions, while scores were approximately one (score=1), mice were randomly divided into four groups including: group 1 was healthy (without any treatment), group 2 was administered PBS, group 3 was administered UCB-MSCs and group 4 was administered MDNPC.

There were six mice in each group. Then, group 3 was injected with 500,000 UCB-MSCs via the tail vein on days 14 and 21 after immunization. In addition, group 4 was injected with 500,000 MDNPC via the tail vein on days 14 and 21 day after immunization. We

Table 1. The number of amplification cycles, sequences, and the amplicon size of the specific primers designed for assessing the expression of neural markers.

<table>
<thead>
<tr>
<th>Markers</th>
<th>Forward primer</th>
<th>Reverse primer</th>
<th>Amplilicon size(bp)</th>
<th>Cycles</th>
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<tr>
<td>MAP2</td>
<td>AGT TCC AGC AGC GTG ATG</td>
<td>CAT TCT CTC TTC AGC CTT CTC</td>
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<td>35</td>
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<td>GFAP</td>
<td>GCA GAC CTT CTC CAA CCT G</td>
<td>ACT CCT TAA TGA CCT CTC CAT C</td>
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<td>MBP</td>
<td>ACC CCG TAG TCC ACT TCT TC</td>
<td>ACT CCC TTT AAT CCC TTG TG</td>
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<tr>
<td>Nestin</td>
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<tr>
<td>β-actin</td>
<td>CTT CCT TCC TGG GCA TG</td>
<td>GGG GTG TTT GGG GAT GTC CAC</td>
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<td>35</td>
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</tbody>
</table>
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recorded the clinical score and weight of EAE mice treated with UCB-MSCs, MDNPC, and PBS for 56 days after immunization. Finally, we analyzed disease status with H&E in brain sections of four groups of the mice.

**Histopathology Analysis**

Fifty-six days after immunization, the mice were deeply anesthetized with ketamine/xylazine (5/1). Following intracardiac fixative perfusion, the brain was fixed in 4% paraformaldehyde for 6 hours. Tissues were treated with ethanol and xylene according to the routine protocol, and 5 µm sections were stained with H&E to detect perivascular inflammatory infiltrates. In each animal, six random sections of the brain were photographed using Olympus DP70 digital camera and the acquisition software Image-Pro Plus.

**Statistical Analysis**

Statistical analysis was performed using SPSS 11.5 software. Average EAE scores were analyzed by Kruskal–Wallis test to determine the difference among all dose-treated groups, and Mann–Whitney U test was used to determine the significant difference between treatment and control groups. One-way ANOVA was also applied to test the differences among all groups. Data were presented in the form of mean±SD and p<0.05 was considered as significant.

**RESULTS**

**Characterization and Differentiation Assay of UCB-MSCs**

UCB-MSCs were first characterized for the expression of specific markers by flow cytometry. MSCs were positive for CD105 (90.9±8.1, n=3), while they were negative for hematopoietic marker of CD34 (2.6±2.5, n=3) (p<0.05) in comparison to isotype control (Figure 1).

**Morphological Changes on Differentiation of UCB-MSCs into Neural Progenitor Cells**

In this study, confirmed UCB-MSCs were used to estimate the capacity of differentiation into neuronal progenitor cells. UCB-MSCs were induced for 6 days and their morphological change was confirmed. Before induction, UCB-MSCs began to change morphologically into a spindle shape. Following neural induction, UCB-MSCs were progressively involved in a long process of extension and neuron-like morphology (Figures 2A and B).

**Neural–specific Genes Studies**

Neuronal-specific genes, such as GFAP, MAP2, MBP and nestin, were not expressed in neuronal undifferentiated UCB-MSCs (control group) by qPCR. However, mRNA of these markers was expressed in neuronal differentiated UCB-MSCs. Interestingly, expression level of GFAP protein was higher than that of other genes in differentiated UCB-MSCs.

**MDNPC Improved Body Weight in EAE Mice**

To investigate the effect of MDNPC on improving body weight, we monitored the weight of the animals every 2 days. Body weight in mice that received MDNPC in intravenous routes was significantly higher (p<0.05) as compared to EAE mice treated with PBS.
Figure 2. A, B: Neurogenic differentiation capacity of UCB-MSCs. Morphological appearance of neural differentiated human cord blood mesenchymal stem cells (UCB-MSCs). (A) Before differentiation, UCB-MSCs showed fibroblast-like shaped cells. (B) After UCB-MSCs showed the progressively acquire neuron-like morphology, a large nucleus, and long processes, magnification= x100

as well as EAE mice treated with MSCs. At days 34-56, body weight in healthy mice was significantly increased ($p<0.05$) compared to MDNPC, MSC and EAE without cell (Figure 3). At days 14-22, body weight in PBS and MSCs groups decreased significantly ($p<0.05$) in comparison to MDNPC and healthy groups. At days 44-56, body weight in PBS group was decreased significantly ($p<0.05$) compared to MDNPC, MSCs and healthy groups (n=6 per each group). Substantial loss of body weight in PBS/EAE group was initiated at day 44 (Figure 3).

**MDNPC Alleviate the Severity of Clinical Scores in EAE Mice**

We sought to address whether MSC and MDNPC transplantation through intravenous routes may reduce clinical scores in EAE C57BL/6 mice 56 days post-immunization (DPI). We observed a substantial suppression of the clinical scores of EAE following MDNPC transplantation (Figure 3). Clinical scores in mice that received MDNPC via intravenous routes were significantly lower ($p<0.05$) as compared to EAE mice treated with PBS as well as those treated with MSCs (Fig. 3B). The 56-day observation period was divided into three stages, including the sub-acute (10–30 DPI) and acute phases (30–47 DPI), in which clinical scores in EAE mice treated with PBS and those treated with MSCs are at the maximum, and the chronic phase (48–56 DPI) as a stable feature of EAE disease.

Difference in clinical scores (48–56 DPI) between PBS/EAE and MSCs/EAE animals was not significant. In most C57BL/6 mice, the onset of clinical symptoms occurred 10–12 days after immunization with MOG. In sub-acute phase, on days 10–20 of DPI, there was no significant difference between the average clinical score and maximum clinical score among untreated EAE mice and MDNPC injected groups. While at 22–30 DPI of sub-acute phase, there were significant differences in the average clinical scores and the maximum clinical score between the untreated EAE mice and MDNPC groups ($p<0.05$).

Maximum clinical scores were observed in mice of the PBS group (score 4), MSC group (score 4) and MDNPC group (scored 1.5). Severity of clinical scores in the acute and chronic phases in treated animals (MDNPC group) including intravenous routes was decreased significantly ($p<0.01$) compared to EAE mice without cell transplantation (Figure 3).

**MDNPCs Reduce Leukocyte Infiltration into the Brain during Chronic EAE**

To evaluate the effect of MSCs and MDNPC on abrogating inflammatory lesions in the brain, the mice were sacrificed for post-mortem analysis, and leukocyte infiltration was assessed by counting the number of cells per 100μM slide stained with H&E. All accumulated cells in the same region were counted in every slide. Mean number of cells in eight slides per
Figure 3. MSCs-NPCs (MDNPCs) injections improve EAE disease scores and weight of EAE mice. (A) Daily average EAE weight of mice treated with intravenously injected PBS. (B) Daily average disease score of mice treated with intravenously injected PBS. (C) Daily average EAE weight of mice treated with intravenously injected MSCs-NPCs. (D) Daily average disease score of mice treated with intravenously injected MSCs-NPCs.
Figure 4. Decline of brain inflammation representative images from control normal (A, B) PBS/EAE (C, D) MSCs/EAE (E, F) and MSCs-NPCs/EAE transplanted (G, H) Here were more perivascular cuffing or inflammation around a blood vessel in corpus callosum in PBS brains than in MNPCs transplanted EAE brain Pen vascular inflammatory cell infiltrated in H&E stained brain tissue (A, C, E 100x Magnification) and (B, D, F, H 200x Magnification). Bar 100µM Quantification was performed on 3 sections per animal and 6 animals per group. Average colonies of cells in PBS/EAE groups was 3 including 62 cells per 100μM filed of slides, average colonies of cells in EAE with MSCs transplantation was 2 including 41 cells per 100μM field of slides; average colonies of cells in EAE with MNPCs transplantation was 1 including 23 cells per 100μM field of slides. Cell density was significantly lower (p<0.01) in treated mice as compared to EAE group without cell injection. In mice injected MNPCs, cell infiltration was decreased significantly (p<0.05) compared to animals injected UCB-MSCs.

DISCUSSION

Consistent with previous studies, neural lineage potential of MDNPC was evidenced by up-regulation of neural-specific genes such as GFAP, MBP, MAP-2, and nestin. One of the key aims of this study was to compare the preclinical efficiency of UCB-MSCs versus MDNPC. MDNPC are derived from umbilical cord blood MSCs, and are thus a convenient source of stem cells with neural progenitor properties to be used in CNS repair strategies. Based on previous studies, MDNPC are not only involved in immune responses but contribute to other important physiological functions in tissues, including the nervous system. In this study, following intravenous transplantation of MDNPC, reduction in leukocyte expression was observed in the brain. In addition, we demonstrated that transplantation of MDNPC versus UCB-MSCs with one-week interval resulted in significant amelioration of clinical and histopathological features of EAE.

Clinical studies have been conducted in order to study the integrity and efficiency of mesenchymal cells in MS patients. Nevertheless, contradictory results have sometimes been observed on therapeutic
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application of MSCs in EAE or other diseases. It could be concluded that MSCs have a high therapeutic potential in treating MS but their treatment efficacy need to be increased, so different strategies could be used. In this work, to study the integrity of MSCs and increase their transplantation efficiency, it was decided for the first time to study the impact of protective transplantation of MDNPC in an EAE.

In this study, after induction of EAE, the mice showed the first sign of EAE (reduced tone of tail) on day 11. After transplantation of 1000000 MDNPC through intravenous route from day 21, the scores remained fixed and were gradually reduced; causing improved clinical scores and movement of mice limbs. In our study, in terms of score, transplantation of MSC did not cause significant reduction compared to PBS and MDNPC groups. These findings are consistent with the findings of Mojadadi et al’s study. Body weight in PBS/EAE group on days 12–30 and 48–56 (Figure 3) was decreased significantly (p<0.05) compared to MDNPC/EAE and healthy groups, whereas no major difference was observed between the PBS/EAE and MSCs groups. Loss of body weight in PBS/EAE group was initiated substantially on day 30, which was consistent with Mojadadi et al’s results.

In our study, by studying the weight and clinical symptoms using linear regression and ANOVA in different groups, it was revealed that the mice injected with MDNPC showed improvement in clinical scores and weight gain. In this study, following intravenous transplantation of MDNPC, reduction in leukocyte expression and inflammation was seen in the brain. The exact mechanisms by which MDNPC exert clinical benefit, inhibit progression and cause relative improvement in EAE need further investigations. The expression rate and number of leukocyte were determined based on similar study experiences and pathologists’ view in sections of different mice groups. Through H&E staining of brain parenchyma and paravascular areas in PBS/EAE group, cell expression (Figure 4), lymphocyte gathering and increase in reactive astrocytes were observed in MDNPC/EAE group. As expected, the number of lymphocytes expressed around the paravascular showed a significant reduction compared to PBS/EAE group, which was consistent with the clinical findings in the group under study.

In this study, angiogenesis, another sign for vascularization and improvement in disease procedures, were seen in the brain parenchyma in MDNPC/EAE group compared to other groups. MDNPC are likely to be converted from TH1 to TH2 through change in cytokine profile, shifting the cell balance toward anti-inflammatory conditions in EAE mice and inhibiting trophic factor expression, resulting in reduction of inflammation in CNS mice (EAE). In one of the previous experiments, it was shown that transplantation of BM-NPC has the potential for therapeutic application in MS. Thus, based on this study, it seems that MDNPC are more efficient in treating EAE compared to MSCs, and this finding is consistent with previous works.

In conclusion, our results suggest that (1) UCB-MSCs can exhibit neuronal differentiation potential under specific condition in vitro. (2) Transplanted MDNPC lowered clinical score and reduced CNS leukocyte infiltration compared to control group. (3) These results clearly support the protective capacity of neural progenitor cell transplantation for MS treatment in the future but further investigations are needed.

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