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For the Degree of Doctor of Veterinary Medicine

Human adipose tissue derived mesenchymal stem cells ameliorate experimental autoimmune encephalomyelitis in Lewis rats.

Myung Soon Ko

Department of Veterinary Medicine,

GRADUATE SCHOOL,
JEJU NATIONAL UNIVERSITY

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Myung Soon Ko
(Supervised by Professor Kyoung-Kap Lee)

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This thesis has been examined and approved.

Thesis director, Taekyun Shin,
Prof. of College of Veterinary Medicine

Young-Min Yun, Prof. of College of Veterinary Medicine

Jeong-Chan Ra, PhD, CEO of RNLBIO Co., Ltd.

Gonhyung Kim, Prof. of College of Veterinary Medicine,
ChungBuk National University

Kyoung-Kap Lee, Prof. of College of Veterinary Medicine

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JEJU NATIONAL UNIVERSITY

CONTENTS

Abstract

The effects of human adipose tissue-derived mesenchymal stem cells (hAdMSCs) were examined on clinical symptoms of experimental autoimmune encephalomyelitis (EAE) in Lewis rats. EAE was induced in Lewis rats immunized with bovine myelin basic protein (bMBP) and complete Freund's adjuvant supplemented with *Mycobacterium tuberculosis* H37Ra.

Cells were administered to rats on day 7 post immunization (PI) to evaluate the dose-dependent clinical and pathological effects of hAdMSCs in acute EAE. Vehicle-treated control rats received normal saline each time. Administration of hAdMSCs significantly ameliorated the duration of paralysis in EAE rats at days 7 PI once time or 1 and 7 PI twice time treated compared with vehicle-treated control rats ($p<0.05$).

Clinical signs were significantly reduced in hAdMSC-treated EAE rats (n=8) at days 1 and 7 PI compared to EAE control rats (n=7). Treatment with hAdMSCs reduced the encephalitogenic response and decreased infiltration of inflammatory cells in the EAE-affected rats. Relatively few inflammatory cells were identified in the spinal cords of hAdMSC-treated EAE rats. Quantitative real time-polymerase chain reaction (qRT- PCR) analysis revealed that interferon-ɣ (IFN-ɣ) expression was significantly increased ($p<0.01$); tumor necrosis factor-α (TNF-α) and interleukin-17f (IL-17f) mRNA expression was not significantly increased and transforming growth factor-β1 (TGF-β1) expression was similar for each group. Interleukin-10 (IL-10) and Indoleamine

2,3-dioxygenase (IDO) mRNA levels were significantly increased ($p<0.05$) in the hAdMSC-treated group. Further, hAdMSCs were detected in the spinal cord and the splenic tissue on day 7 after hAdMSC injection.

The ameliorative effect of hAdMSCs on clinical paralysis in EAE-affected rats may be mediated, in part, by the suppression of the autoreactive T cell response and the dominance production of anti-inflammatory cytokines such as IL-10 and IDO.

Results from the present study suggest mesenchymal stem cells derived from human adipose tissue may provide neuroprotection and immunomodulation in disease such as EAE.

Key words : human adipose tissue derived mesenchymal stem cells, EAE, Interleukin -10; Indoleamine 2,3-dioxygenase

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List of abbreviation

bMBP	Bovine myelin basic protein
CFA	Complete Freund's adjuvant
CNS	Central nervouse system
EAE	Experimental autoimmune encephalomyelitis
Foxp3	Forkhead box p3
GMP	Good manufacturing practice
GVHD	Graft-versus-host disease
hAdMSCs	Human adipose tissue derived mesenchymal stem cells
IDO	Indoleamine 2,3-dioxygenase
IFN-γ	Interferon-γ
IHC	Immunohistochemistry
IL	Interleukin
MOG	Myelin oligodendrocyte glycoprotein
MS	Multiple sclerosis
MSC	Mesenchymal stem cell
PI	Post immunization
PLP	Proteolipid protein
qRT-PCR	Quantitative real time-polymerase chain reaction
SEM	Standard error of the mean
TGF-β	Transforming growth factor-β
TNF-α	Tumor necrosis factor-α
T_{reg}	Regulatory T cell

1. Introduction

Mesenchymal stem cells (MSCs) may act as potent immunomodulatory agents in a variety of clinical and experimental scenarios. MSCs are potent immunomodulatory and multipotential nonhemopoietic progenitor cells capable of differentiation into multiple lineages of cells, including nerve cells. They escape alloantigen recognition because of low immunogenicity and may inhibit both *in vitro* and *in vivo* immune responses. The inhibiting effects are related to immunomodulation of dendritic cells, T-cell and natural killer cells (Aggarwal *et al.*, 2005; Sotiropoulou *et al.*, 2006; Nauta *et al.*, 2007). Such properties classify MSCs as promising candidate cells in the prevention of rejection in organ transplantation and autoimmune disease treatment (Le Blanc *et al.*, 2004; Joo *et al.*, 2010) and in autoimmune disorders (Zappia *et al.*, 2005). A recent case report suggested that systemic MSCs infusion resulted in suppression of severe treatment-resistant acute Graft-Versus-Host Disease (GVHD) after allogeneic bone marrow transplantation (Le Blanc *et al.*, 2004). In addition, mesenchymal stem cells derived from umblical cord blood, bone marrow and adipose tissue have been useful in the treatment of systemic lupus erythematosus, Sjogren's syndromem autoimmune hemolytic anemia, progressive systemic sclerosis and polymyositis (Liang *et al.*, 2009).

Multiple sclerosis (MS) is an immune-mediated demyelinating inflammatory disease of the central nervous system associated with severe physical and cognitive impairment. It is characterized by morphologic hallmarks including inflammation,

demyelination, oligodendrocyte loss, and axonal and neuronal damage. Inflammation leads to multifocal demyelination within the white matter; this affects nerve conduction speeds and results in significant functional deficits (Torgerson *et al.*, 2006).

Experimental autoimmune encephalomyelitis (EAE) is the animal model of human autoimmune central nervous system (CNS)-demyelinating multiple sclerosis (Olsson *et al.* 1995; Wong *et al.,* 1999). It is a T cell-mediated autoimmune disease of the CNS characterized by demyelination and accumulation of cellular infiltrates into the subarachnoid space early disease stages; peak (symptomatic) stages are characterized by microglia and astrocyte activation (Shin *et al.*, 1995; Martin *et al.*, 1995).

Disease progression and severity of clinical signs are associated with the extent of infiltrating inflammatory cells in EAE lesions (Ahn *et al.*, 2001) and the involvement of different cell types including Th1, Th2, Th17, cytotoxic T cells, B cells and regulatory T cells in the inflammatory processes of both MS and EAE (Holmøy *et al.*, 2008). Pro- and anti-inflammatory cytokines, including tumor necrosis factor-α (TNF-α), interferon-ɣ (IFN-ɣ), Transforming growth factor-β1 (TGF-β1), interleukin-1β (IL-1β), interleukin-4 (IL-4), interleukin-6 (IL-6), and interleukin-10 (IL-10) are involved in the pathophysiology of MS and EAE (Imitola *et al.* 2005; McGeachy *et al.*, 2005). The primary target for amelioration of EAE and MS is immunomodulation by Th1, Th17, cytotoxic T cells, B cells and regulatory T cells (Bettelli *et al.*, 1998; Ponomarev *et al.*, 2004; Anderson *et al.*, 2004; O'Neill *et al.*, 2006; Nauta *et al.*, 2007; Hong *et al.*, 2009; Correale *et a*l., 2010; Venken *et al.*, 2010).

MSCs have been used to treat chronic EAE in myelin oligodendrocyte glycoprotein $(MOG)^{35-55}$-induced experimental autoimmune encephalomyelitis (EAE). MSCs administration prior to disease onset (Zappia *et al.*, 2005) or during adoptive transfer (Gerdoni *et al.*, 2007; Constantin *et al.*, 2009) ameliorated EAE in previous studies. In addition, intravenous administration of human MSCs in proteolipid protein (PLP)-induced EAE resulted in functional neurological recovery and decreased demyelination in mice, possibly through the elevation of neuroprotective factor expression and changes in cytokine and oligodendrocyte proliferation (Zhang *et al.*, 2005; Bai *et al.*, 2009).

Stem cell therapy has recently been developed in the treatment of autoimmune disease and MSCs have been proposed as a possible MS treatment (Riordan *et al.*, 2009) Further, MS patients have impaired MSCs function compared to healthy donors; this difference resulted in MSCs transplantation as part of MS therapy (Mazzanti *et al.*, 2008; Burt *et al.*, 2009).

Human adipose tissue-derived MSCs (hAdMSCs) are an abundant, easily accessible, and appealing source of donor tissue for autologous cell transplantation (Fraser *et al.*, 2006; Parker *et al.*, 2006). In addition, hAdMSCs have a significantly higher frequency of occurrence in adipose tissue than bone-marrow derived stem cells (Fraser *et al.*, 2006; Parker *et al.*, 2006). However, the effects of hAdMSCs on acute EAE have not been extensively studied to examine the possibility for stem cell-based therapy in acute inflammatory CNS diseases.

The objective in this study was to investigate the effects of

hAdMSCs on disease progression in the present study and also examined cytokine and regulatory T (T_{reg}) cells in the spinal cords and lymphatic tissues in Lewis rats with acute EAE.

2. Materials and Methods

2.1. Animals

Lewis rats were purchased from Japan SLC, inc (Shizuoka, Japan). Seven-week-old female rats were used for all experiment. All experiments were conducted in accordance with the Jeju National University Guide for the Care and Use of Laboratory Animals.

2.2. EAE induction

The footpad of both hind feet of rats in the EAE group were injected with 100㎕ of emulsion containing equal parts of bovine myelin basic protein (bMBP, 1mg/mL) and complete Freund's adjuvant (CFA) supplemented with *Mycobacterium tuberculosis* H37Ra (5mg/mL; Sigma, St Louis, MO). Body weights of all rats were measured daily and rats were observed for clinical signs of EAE.

EAE progression was divided into eight clinical stages: grade 0 (G.0), no signs; G.0.5, mild floppy tail; G.1, complete floppy tail; G.2, mild hind limb paraparesis; G.3, severe hind limb paraparesis; G.4 tetraparesis; G.5, morbund condition or death; R.0, recovery.

2.3. Cell preparation and culture of human adipose tissue derived mesenchymal stem cells

hAdMSCs were donated from RNLBIO (Seoul, Korea). Isolation of hAdMSCs was performed as previously described (Ra *et al.*, 2008).

Briefly, human adipose tissue from simple donor liposuction procedures were obtained with informed consent and approved according to the procedures of the institutional review board.

The subcutaneous adipose tissues were digested with 4㎖ RTase (RNLBIO, Seoul, Korea) per 1g fat under gentle agitation for 60 min at 37℃. The digested tissues were filtered through a 100 ㎛ nylon sieve to remove cellular debris and were centrifuged and pelleted at 1500 rpm. The pellet was resuspended in RCME (RNLBIO, Seoul, Korea) containing 10% fetal bovine serum (FBS). The cell suspension was re-centrifuged at 1500 rpm for 5 min. The supernatant was discarded and the pellet was collected. The cell fraction was cultured over night at 37℃/5% CO2 in RCME containing 10% FBS. Cell adhesion was examined under an inverted microscope 24 hours later. Non-adherent cells were removed after 24 hours and were washed with phosphate-buffered saline (PBS). The cell medium was then changed with RKCM (RNLBIO, Seoul Korea) containing 5% FBS. Cells were maintained over four or five days until they became confluent, as represented at passage 0. Cells were subculture-expanded in RKCM until passage 3 at 90% confluence.

MSC immunophenotypes were analyzed using a FACS

calibur flow cytometer. Every harvest of MSCs revealed a homogenou spopulation of cells with high CD73 and CD90 expression and minimal expression of CD31, CD34 and CD45. Cell viability evaluated by Trypan blue exclusion prior to shipping was greater than 95%. No evidence of bacterial, fungal, or mycoplasmal contamination was observed in tested cells prior to shipping. MSC preparation procedures were performed under Good Manufacturing Practice (GMP) conditions (RNLBIO, Seoul, Korea).

Flow cytometry demonstrated the presence of $CD29^{+}$, $CD31^{-}$, $CD34^{-}$, $CD44^{+}$, $CD45^{-}$, $CD73^{+}$, $CD90^{+}$, $CD105^{+}$ and $HLADR^{-}$ hAdMSCs, suggestive of a MSC phenotype.

2.4. hAdMSCs Treated protocols

The cells were administered at 7 days postimmunization (PI) in three doses (1×10^{6} cells, 5×10^{6} cells and 1×10^{7} cells, all in 500$\mu\ell$ normal saline) to evaluate the efficacy of hAdMSCs in acute EAE. The hAdMSCs (5×10^{6}) were injected intravenously on days 1 and 7 PI and on days 7 and 10 PI to assess transplantation-time dependent effects; hAdMSCs were resuspended in 500$\mu\ell$ normal saline for each injection. Tail vein injections of hAdMSCs (1×10^{7}cells) were performed on days 7 and 10 PI. Vehicle-treated rats received 500$\mu\ell$ normal saline at each injection.

2.5. T cell culture and proliferation assay

Spleen mononuclear cells (MNCs) from rats treated with hAdMSCs on days 1 and 7 PI (n=3) and vehicle treated rats (n=3) described above were dissociated through a 70㎛ cell strainer (BD Falcon, MA, USA) to singularize splenocyte and to remove connective tissue, then washed with PBS and centrifuged at 1500 rpm and 4℃ for 5 min. Lysis of erythrocytes was performed by suspending the pelleted splenocytes in ACK Lysing Buffer (Cambrex) for 15 min in dark room. Cells were washed twice with PBS and suspended in culture medium containing Dulbecco''s modified Eagle''s medium (Gibco, Paisley, UK), supplemented with 1% (v/v) minimum essential medium (Gibco), 2 mM glutamine (Flow Laboratory, Irvine, CA), 50 IU/㎖ penicillin, 50 ㎎/㎖ streptomycin and 10% (v/v) fetal calf serum (Gibco). MNCs were isolated and incubated (4×10^5 MNCs with 200 ㎕ of culture medium) in 96-well, round-bottomed microtiter plates (Nunc, Copenhagen, Denmark). Crude bovine MBP was added to wells at final concentrations of 10 ㎍/㎖ bMBP. After 3 days of incubation, the cells were pulsed for 18h with 10 ㎕ portions containing 1 μCi of ^{3}H-methylthymidine (specific activity 42 Ci/mmol; Amersham, Arlington Heights, IL). The cells were harvested using a Fitermate™ Universal Harvester (Perkinelmer, USA) on glass fiber filters and thymidine incorporation was measured using a Wallac MicroBeta Counter (Perkinelmer, USA). Proliferation was measured form triplicate cultures.

2.6. Quantitative real time PCR analysis of cytokine change

Total RNA was isolated from the sciatic lymph node using TRIzol reagent (Invitrogen) according to the manufacturer's instructions. cDNA was synthesized with the ImProm-Ⅱ™ Reverse Transcription system (Promega, USA) using the oligo (dT) primer. Cytokine mRNA expression was quantitated by real-time PCR using IQ SYBR Green Supermix (Bio-Rad). The PCR was performed with the Bio-Rad Chromo4 real time PCR detector (Bio-Rad). All components of the PCR mix were purchased from Bio-Rad and used according to the manufacturer's instructions. RT-PCR specificity was controlled by the generation of melting curves and agarose gel loading. Cytokine mRNA expression threshold values were normalized to ß-actin expression Data analysis was performed using gene expression analysis for Chromo4 Real-time PCR detection system (Bio-Rad). Primers for target genes and annealing temperatures are shown in Table1.

Table 1. Primer sequences used for qRT-PCR

Gene	Primer Sequence	Product size (bp)	Anneling temperature (℃)
INF-γ	Forward: 5'-ATC GAA TCG CAC CTG ATC ACT A-3' Reverse: 5'-TGT GGG TTG TTC ACC TCG AA-3'	95	62
TNF-α	Forward: 5'-GTG ATC GGT CCC AAC AAG-3' Reverse: 5'-AGG GTC TGG GCC ATG GAA-3'	71	61
IL-17f	Forward: 5'-CTC AGA CTA CCT CAA CCG TTC C-3' Reverse: 5'-GTG CCT CCC AGA TCA CAG AAG-3'	89	63
IL-10	Forward: 5'-CTG TCA TCG ATT TCT CCC TGT GAG-3' Reverse: 5'-TGA GTG TCG CGT AGG CTC CTA TGC-3'	132	64
TGF-β1	Forward: 5'-TGA TAC GCC TGA GTG GCT GTC TTT-3' Reverse: 5'-AAG CGA AAG CCC TGT ATT CCG TCT-3'	87	63
IDO	Forward: 5'-AAG CAC TGG AGA AGG CAC TGT GTA-3' Reverse: 5'-ATC CAC GAA GTC ACG CAT CCT CTT-3'	86	65
Foxp3	Forward: 5'-AGA GTT TCT CAA GCA CTG CCA AGC-3' Reverse: 5'-TGC ATA GCT CCC AGC TTC TCC TTT-3'	136	64
β actin	Forward: 5'-AAG CCA ACC GTG AAA AGA TG-3' Reverse: 5'-ACC AGA GGC ATA CAG GGA CAA-3'	101	63

2.7. Histological examination.

Rats were euthanized on day 14 PI, and spinal cords were separated and dissected. Spinal cord samples were processed for embedding in paraffin wax after fixing in 10% buffered formalin. Paraffin sections were used for hematoxylin-eosin staining and immunohistochemistry.

2.8. Immunohistochemistry for detection of hAdMSCs and the expression of forkhead box p3 (Foxp3) in spinal cord and spleen.

Paraffin-embedded spinal cord sections (5 μm thick) were deparaffinized, treated for 3 min with 0.01 M citrate buffer (pH 6.0) in a microwave for antigen retrieval, and then treated with 0.3% hydrogen peroxide in distilled water for 20 min to block endogenous peroxidase activity. Sections were incubated with 10% normal goat or porcine serum after 3 PBS washes to evaluate the tissue distribution of hAdMSCs with human nuclear antibody (1:200, MAB 1281, clone-235-1, millpore, USA).

Foxp3 expression was detected by immunohistochemistry (IHC) using affinity-purified anti-rat FJK-16s antibody (1:100; eBioscience, Frankfurt, Germany) for 1 h at room temperature.

Immunoreactivity was visualized using the Vector Elite avidin - biotin peroxidase complex (Vector Laboratories). The peroxidase reaction was developed using a diaminobenzidine

substrate kit (Vector Laboratories). Foxp3 sections were counterstained with hematoxylin prior to mounting.

2.9. Statistical analysis

A two-tailed Student's t test was performed to evaluate differences between hAdMSC-treated and control conditions. Data were expressed as mean ± standard error of the mean (SEM) values. Statistical significance was established at p values<0.05.

3. Results

3.1. EAE was treated dose dependently reduced the duration of paralysis after hAdMSCs treatment.

Dose-dependent effects of hAdMSCs were evaluated after intravenous administration to on day 7 postimmunization (PI). The course of EAE paralysis in this group was significantly associated with dose (Table 2). The hAdMSC-treated groups (5×10^6cells and 1×10^7cells) significantly ameliorated the duration of paralysis in EAE rats compared to the vehicle-treated control group($p<0.05$).

Table 2. Dose-dependent effects of human Adipose tissue derived mesenchymal stem cells (hAdMSCs) on the clinical signs of EAE.

	Incidence of EAE (paralysed/total animal)	First onset of paralysis	Average of Max. clinical score	Duration of paralysis (days)
Vehicle control	7/7	12.7±0.36[b]	1.29±0.18	6.14±0.34
1×10^6cells[a]	6/7	12.5±0.34	1.0±0.0	6.5±0.34
5×10^6cells[a]	7/7	13.3±0.36	1.0±0.0	5.0±0.38*
1×10^7cells[a]	7/7	13.1±0.26	1.0±0.0	5.14±0.34*

[a] The hAdMSCs were transplanted at day 7 PI.
[b] Data are expressed as the mean±SEM.
*P<0.05 *vs.* vehicle-treated control (Student's *t*-test)

3.2. EAE was treated time dependently reduced the severity and duration paralysis after hAdMSCs treatment.

The hAdMSCs treatments administered on days 1 and 7 shortened the duration of paralysis compared to vehicle-treated control rats and rats treated with hAdMSCs treated after day 7 PI (Table 3). Clinical signs were significantly reduced in EAE rats in the hAdMSC-treated group (n=7) at days 1 and 7 PI compared to vehicle-treated control rats (n=8), as shown in Figure 1.

Table 3. Treatment time effects of human Adipose tissue derived mesenchymal stem cells (hAdMSCs) on the clinical signs of EAE.

	Days of transplantation	Incidence of EAE (paralysed/ Total animal)	First onset of paralysis	Average of Max. clinical score	Duration of paralysis (days)
Vehicle control		7/7	12.7±0.36[c]	1.29±0.18	6.14±0.34
hAdMSCs transplantation	D 7 PI[a]	7/7	13.1±0.26	1.0±0.0	5.14±0.34*
	D 10 PI[a]	7/7	12.7±0.35	1.0±0.0	5.14±0.46
	D 1 and 7 PI[b]	8/8	13.8±0.72	0.94±0.06*	4.3±0.32*
	D 7 and 10 PI[b]	7/7	12.0±0.22	1.0±0.0	6.85±0.60

[a] The 1×10^7 hAdMSCs were transplanted in single injection.
[b] The total 1×10^7 hAdMSCs was divided in two injection of 5×10^6 cells in 500$\mu\ell$ vehicle.
[c] Data are expressed as the mean±SEM.
*P<0.05 *vs.*vehicle-treated control (Student's *t*-test)

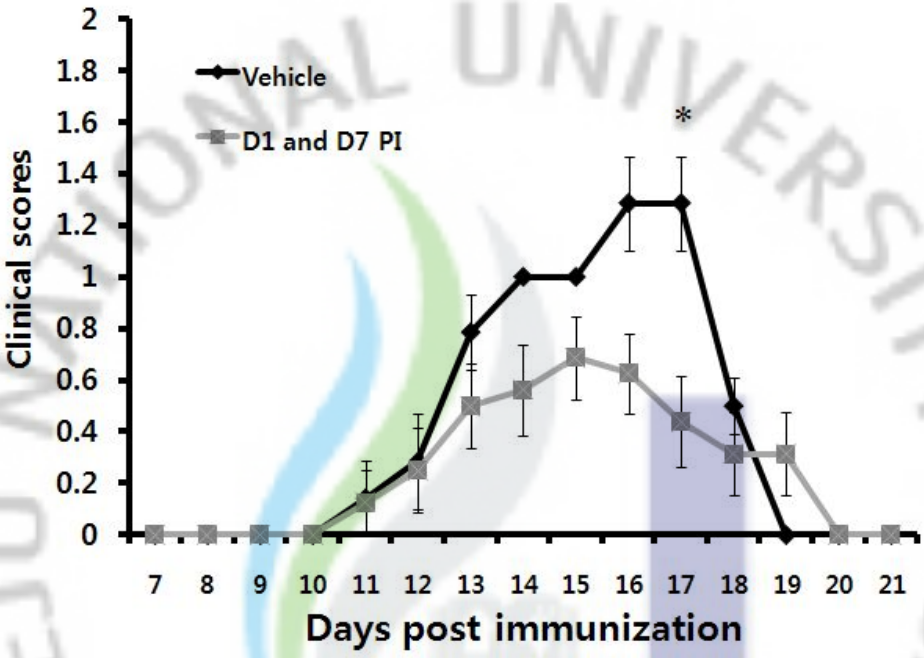

Figure 1. Clinical signs of induced EAE in Lewis rats with or without hAdMSC treatment. EAE was significantly reduced in hAdMSC-treated rats at day 17 PI (n=8).

3.3. hAdMSCs treatment affected the expansion of bMBP-specific T cells.

The study examined whether hAdMSCs treatment affected the expansion of bMBP-specific T cell. As shown in figure 2, the proliferation of antigen-specific T cells increased significantly in vehicle-treated rats. In marked contrast, hAdMSCs-treated rats showed a significant decrease in antigen-specific T cell proliferation in response to bMBP compared with vehicle treated control rats ($p<0.05$).

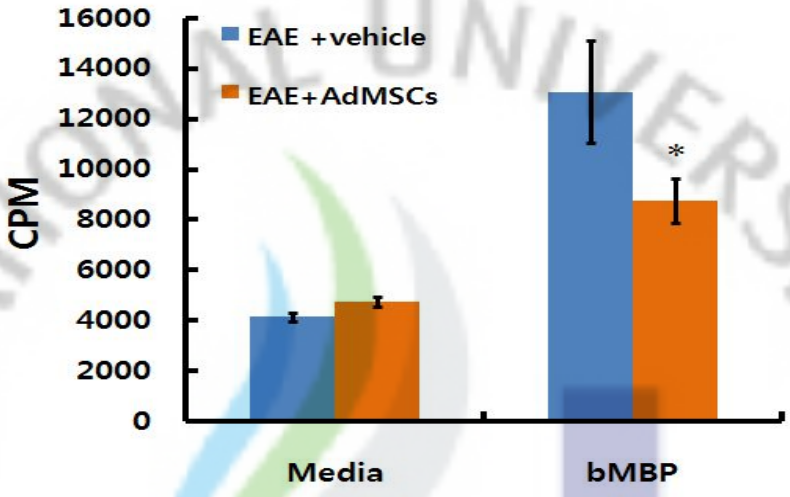

Figure 2. Effects of hAdMSCs on bMBP-reactive T cell responses. Proliferative responses to the antigen (bMBP) were assessed in triplicate wells for each experiment (n=3). Data are mean ± SEM of cpm. Statistical evaluation was performed to compare the experimental groups and corresponding control groups. *$p<0.05$.

3.4. hAdMSCs effected to expression of cytokines in the lymph node.

The qRT- PCR analysis revealed expression of the pro-inflammatory cytokines TNF-α, IL-17f and IFN-ɣ. The responses from hAdMSC-treated animals displayed increased levels of TNF-α, IL-17f and IFN-ɣ compared to responses from vehicle treated control rats. IFN-ɣ expression was significantly ($p < 0.01$) increased, while TNF-α and IL-17f mRNA expression was also increased in the hAdMSCs treated group but not significantly (Figure 3).

The presence of IL-10, IDO and other anti-inflammatory mediators were identified by qRT-PCR. TGF-β expression was similarly increased in each group (Figure 4); however, IL-10 and IDO mRNA levels were significantly increased ($p<0.05$) in the hAdMSC-treated group.

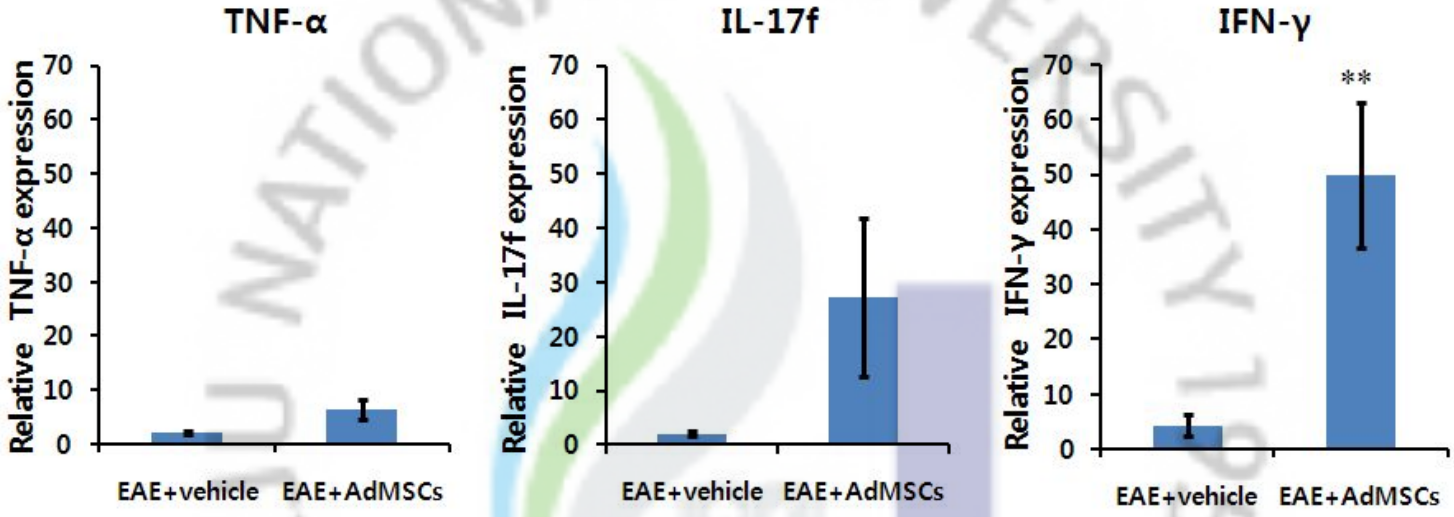

Figure 3. Pro-inflammatory cytokine mRNA expression levels in the lymph nodes at days 14 PI (n=5). Relative mRNA expression for each target gene as measured by qRT-PCR. Values are given as transcript abundance relative to β-actin expression. qRT-PCR data are mean ± SEM values *p<0.05, **p<0.01.

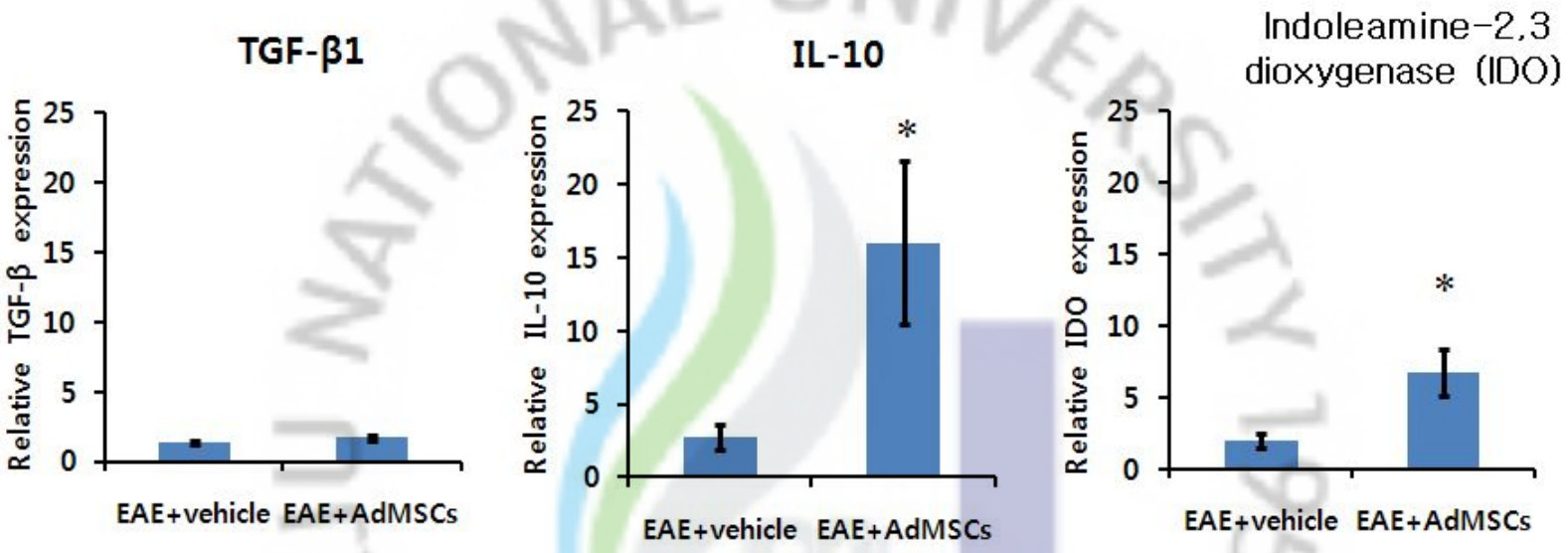

Figure 4. Anti-inflammatory cytokine mRNA expression levels in the lymph nodes at days 14 PI (n=5). Relative mRNA expression for each target gene as measured by qRT-PCR. Values are given as transcript abundance relative to β-actin expression. qRT-PCR data are mean ± SEM values *p<0.05, **p<0.01.

3.5. Expression of Foxp3 in spleen and spinal cord.

The qRT- PCR analysis revealed that Foxp3 mRNA expression was somewhat increased in EAE ratd treated with hAdMSCs, but was not significantly elevated in the lymph nodes on day 14 PI (Figure 5) compared with vehicle-treated control rats.

Foxp3+ cells were similarly identified around infiltrated cells in the spinal cord of EAE rats with hAdMSCs at day 14 PI (Figure 6B), compared to vehicle-treated control rats (Figure 6A). However, the Foxp3+ cells were increased in the spleens of EAE rats treated with hAdMSCs (Figure 6D) compared to the vehicle control rats (Figure 6C).

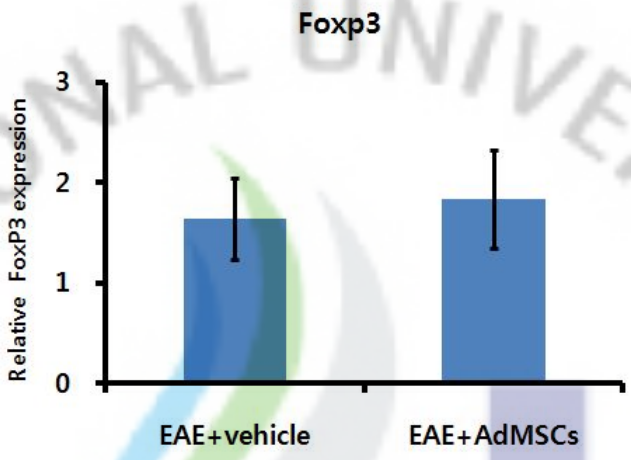

Figure 5. Relative Foxp3 mRNA expression in lymph nodes on day 14 PI (n=5) measured by qRT-PCR. Valves are given as transcript abundance relative to β-actin expression. qRT-PCR data are mean ± SEM values *p<0.05, **p<0.01.

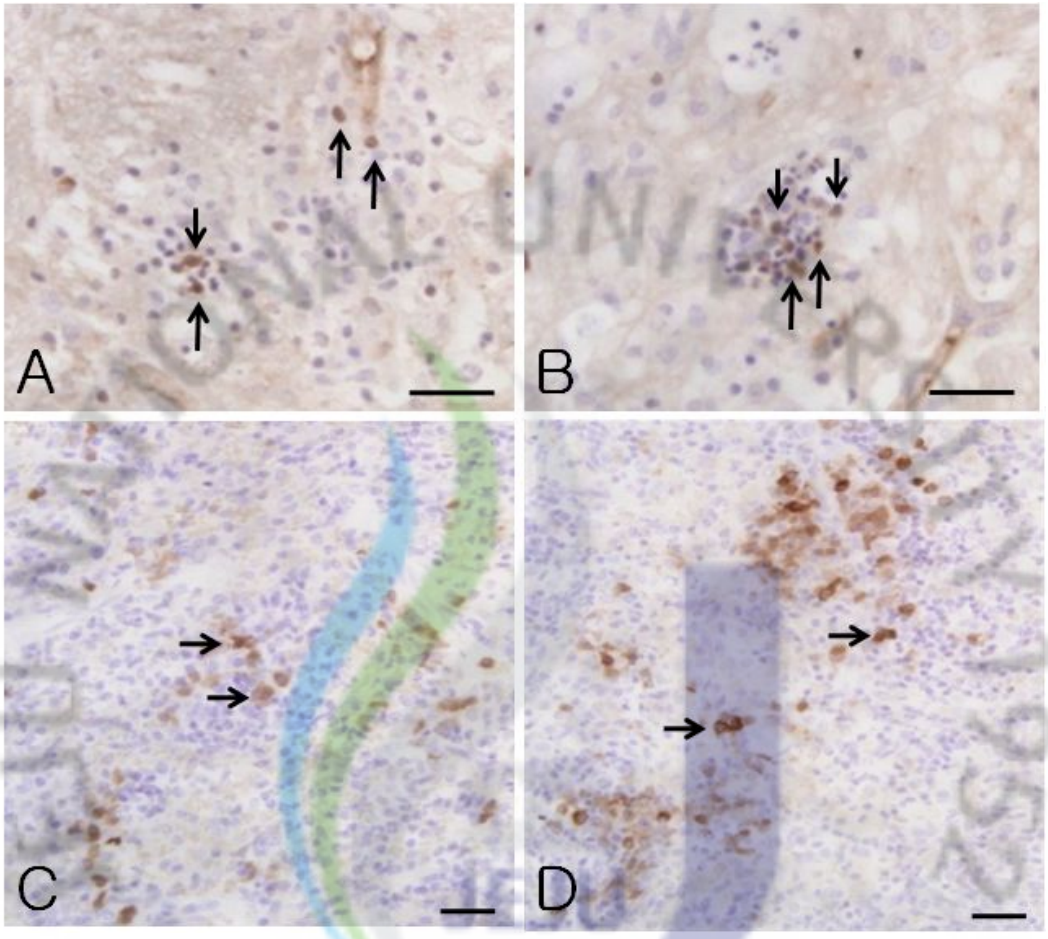

Figure 6. Immunohistochemical staining for Foxp3 in the spinal cords (A and B) and spleens (A and C) of vehicle-treated rats and EAE with hAdMSC-treated rats (B, D) at day 14 PI. Foxp3 positive cells were identified in spinal cord inflammatory cells (A and B, arrows) and spleens (C and D, arrows). Counterstained with hematoxylin. Scale bars=30 μm.

3.6. hAdMSCs reduced the filtration of inflammatory cells in spinal cord with EAE.

Rats were euthanized at days 14 PI under ether anesthesia. Inflammatory cells were identified in the parenchyma of spinal cords with vehicle control rats (day 14 PI). Few infiltrated inflammatory cells were identified in the spinal cords with hAdMSC-treated rats (Figure 7).

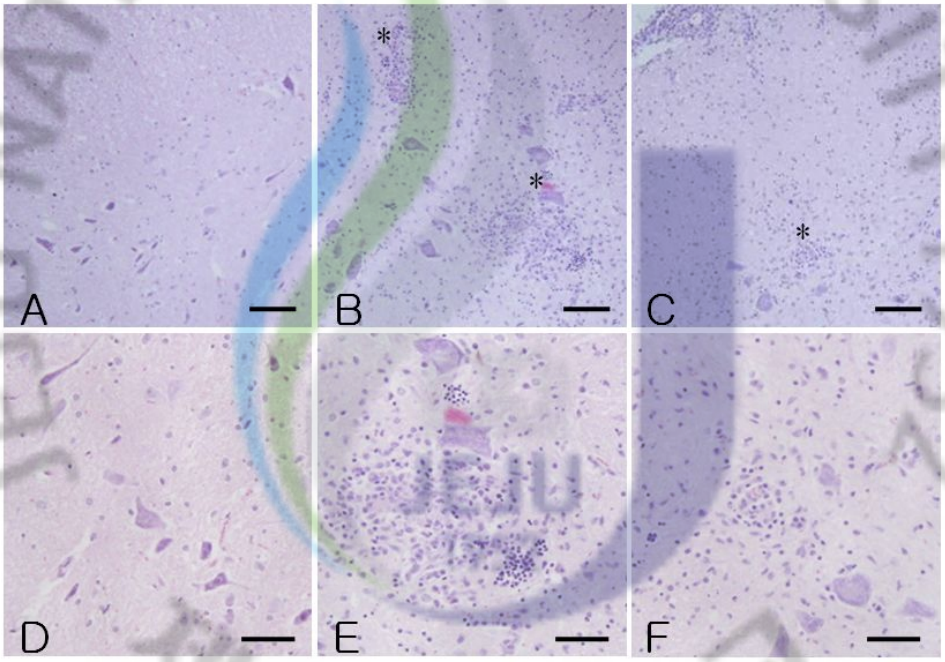

Figure 7. Histopathological findings in the spinal cords of normal (A and D), hAdMSC-treated (C and F) and EAE vehicle (B and E) rats. Inflammatory cells were identified in spinal cord parenchymas of EAE vehicle rats (day 14 PI) (B). There were minimal numbers of inflammatory cells in spinal cords from EAE rats treated with hAdMSCs (C). D, E and F were high magnification of A, B, and C, respectively. Hematoxylin-eosin staining. A-C : scale bars = 100 μm, D-F: scale bar= 50 μm.

3.7. Migration of intravenous hAdMSCs to spinal cord and spleen.

The potential for MSC migration to the spleen and spinal cord were examined to elucidate the *in vivo* immunomodulatory mechanisms of hAdMSCs. Immunohistochemical hAdMSC staining on day 7 used human nuclear antibody (MAB 1281) in the spinal cords (Figure 8A) and the spleen (Figure 8B). Treated hAdMSCs were detected in the parenchyma.

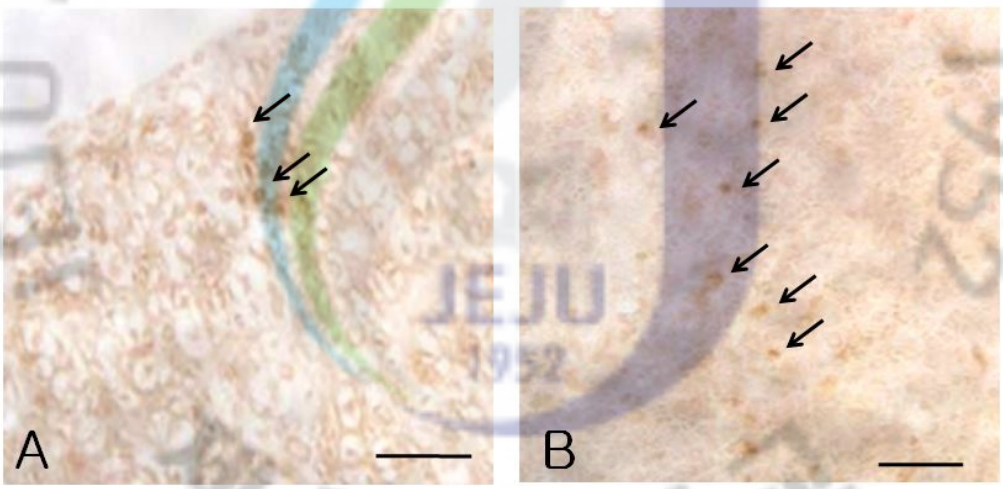

Figure 8. Immunohistochemical staining for hAdMSCs using human nuclear antibody in spinal cord (A) and spleen (B). Treated hAdMSCs were detected in the parenchyma. Scale bars=30 μm.

4. Discussion

This study examined the effects of hAdMSCs in actively-induced Lewis rat EAE to determine whether hAdMSCs ameliorated the clinical signs of rat EAE. These results suggested that administration of hAdMSCs during EAE induction (days 1 and 7 PI) ameliorated EAE paralysis compared to vehicle-treated controls; however, these effects were not noted during the effector stage of EAE (days 7 and 10 PI). This suggests the potential for therapeutic intervention in acute inflammatory CNS diseases in the early period of transplantation in a dose-dependent manner.

Ex vivo analysis of spleen mononuclear cells (MNCs) from rats treated with hAdMSCs showed a significant reduction of proliferation in the presence of bMBP when compared with control rats, indicating that hAdMSCs could alter myelin-specific T cell and their autoreactivity taken together, these data indicate that the capacity of hAdMSCs to suppress the encephalitogenic T cell response may underlie its clinically beneficial effect in EAE.

The cytokine responses in EAE rats after hAdMSCs treatment were determined. Pro-inflammatory cytokines, including TNF-α, IL-17f and IFN-γ increased in rat treated with hAdMSCs compared to vehicle-treated EAE controls with improved clinical scores. In addition, IL-10 and IDO expression, which has a critical role in EAE regulation, was significantly increased in the hAdMSC-treated group.

Previous studies have suggested that the pro-inflammatory

cytokine TNF-α affects acute EAE pathogenesis (Farias *et al.*, 2007) and that the increase in TNF-α was correlated with increased number sinfiltrating inflammatory cells in the spinal cord (Ahn *et al.*, 2001). IL-17f is also increased in lymphocytes derived from mice with EAE (Zhang *et al.*, 2003); the IL-17f immune pathway is may play an important role in the disease process (Komiyama *et al.*, 2006; Kleinschek *et al.*, 2007).

Several studies have demonstrated that hMSCs can mediate suppression of T-cell proliferation by secreted factors such as immunosuppresive cytokine TGF-β or IL-10 (Tse *et al.*, 2003). Recent studies have demonstrated that MSCs are strongly immunosuppressive in *vitro* and *in vivo* (Munn *et al.*, 1998; Meisel *et al.*, 2004; Krampera *et al.*, 2006; Ryan *et al.*, 2007; Yoo *et al.*, 2009; Prasanna *et al.*, 2010). hAdMSCs stimulate the production of IL-10 and IDO, which is important in the recovery from autoimmune diseases, including EAE (Bettelli *et al.*, 1998; Cua *et al.*, 1999; O'Neill *et al.*, 2006; Fitzgerald *et al.*, 2007). IFN-ɣ induced IDO production by MSCs was important in T-cell immunosuppression (Ryan *et al.*, 2007; Meisel *et al.*, 2004). The suppressive effect of MSCs on T-cell proliferation requires the presence of IFN-ɣ and/or TNF-α, which acts by enhancing the IDO activity of MSCs. Non-stimulated MSCs did not secrete IFN-ɣ, which is correlated with the immunomodulatory properties of MSCs. Indoleamine 2,3-dioxygenase (IDO) expression was increased in MSCs treated with IFN-ɣ and/or TNF-α (Yoo *et al.*, 2009).

These findings collectively suggest that the amelioration of rat EAE with treated hAdMSCs in this study is secondary to

induction of IFN-ɣ, IDO and IL-10 expression and immunmodulation by hAdMSCs.

The results show that hAdMSCs produce a benefical effect in acute EAE through the suppression of the autoreactive T cell response and the dominance production of anti-inflammatory cytokines such as IL-10 and IDO.

5. Conclusion

In summary, the practical advantages of using hAdMSCs, the clinicopathologic efficacy, the ability of hAdMSCs to function as both pro- and anti-inflammatory mediator and the possible inhibition of inflammatory cell transmigration into the CNS of EAE-induced rats.

The data in this study show that hAdMSCs have relevant therapeutic potential in an animal model of acute MS and might provide the scientific basis that hAdMSCs represent a valuable tool for stem cell - based therapy in acute inflammatory of the CNS.

자기면역성 뇌척수염에서 사람 지방유래 중간엽 줄기세포의 완화효과

(지도교수 : 이 경 갑)

고 명 순

제주대학교 수의학과

Experimental Autoimmune Encephalomyelitis (EAE)는 인간의 다발성경화증에 대한 연구에 널리 이용되는 동물실험모델로서, EAE는 blood-brain-barrier (BBB)가 소실되어, 질병의 초기 단계에는 T-cell과 대식세포와 같은 염증세포들이 지주막공간으로 이동하여 침윤되며, 질병의 최고 발현기에는 microglia와 astrocyte들이 활성화되는 특징을 보인다. 질병의 임상적 차이는 생체내 자가 면역반응에 크게 영향을 받으며, 신경세포의 손상을 일으켜 장애를 영구화하는 질환이다.

중간엽 줄기세포는 모든 조직으로 분화능과 면역조절능을 지닌 세포로 자가면역성 신경손상질환의 치료 가능성에 대한 많은 연구가 이루어지고 있다. 특히, 자가면역성 뇌척수염에 대한 중간엽줄기세포의 면역조절능에 대해 최근 많은 연구결과들이 보고되고 있는데, 뇌척수염을 일으킨 실험동물에 골수유래중간엽줄기세포를 투여시 생체내에서 Th2-polarized response을 유도하여 TGF-β와 IL-6의 분비 양상의 변화로 염증세포와 탈수초반응을 완화시켜서 임상증상이 완화되었으며, 신경보호물질 분비양상의 변화로 신경세포를 보호한다고 보고하고 있다. 지방줄기세포의 EAE에 대한 항염증효과와 신경보호 작용에 대한 보고로는, 만성 EAE 모델에서의 murin지방유

래줄기세포를 마우스에 투여한 결과, 예방 치료에 효능이 있었으며, 투여한 murine 지방유래줄기세포가 병변부위인 척수에 분포됨을 확인하였다.

이상의 연구보고는 만성 뇌척수염질환동물에서의 임상적 완화 및 분포에 대한 보고로써, 중간엽 줄기세포가 생체내에서 자가면역성 신경손상으로부터 신경세포를 보호하는 기전과 비장과 림프기관에서의 면역관련 세포 및 싸이토카인 분비의 조절에 대한 기전, 병변조직에서의 세포의 변화에 대한 보고는 미비하다. 또한, 급성형 자가면역성 뇌척수염에서의 사람지방유래 중간엽줄기세포의 작용에 대한 보고는 없다.

그러므로, 본 연구에서는 급성자가면역성 척수염 동물모델에서 사람지방유래 중간엽 줄기세포의 임상적 효과, 병변조직에서의 염증세포의 침윤양상, 사이토카인의 변화와 정맥내 투여한 사람지방유래 중간엽줄기세포의 척수병변부위 분포여부를 살펴보았다.

사람지방유래 중간엽 줄기세포의 용량의존적 효과는 면역후 7일째 투여시 5×10^6 cells 이상의 세포를 투여한 군에서 대조군에 비해 마비기간이 유의성있게 단축되었다. 또한 투여시기에 따른 변화에서는 면역 후 1일과 7일째에 5×10^6 cells을 2회 투여한 군에서 대조군, 면역 후 7일과 10일째에 5×10^6 cells을 2회 투여한 군 및 면역 후 10일째 1×10^7 cells을 1회 투여한 군에 비해 마비기간이 유의성 있게 단축되었고, 마비정도도 유의성 있게 완화되었다.

사람지방유래 중간엽 줄기세포를 투여한 쥐의 비장에서 분리한 T cell에 항원인 bovine MBP로 자극 후, T cell 증식 배양실험을 한 결과, 대조군에 비해 유의적으로 bMBP-specific T cell의 증식이 억제되었다.

면역 후 14일 즉, 사람지방유래 중간엽 줄기세포를 투여한 7일 후, 대조군과 사람지방유래 중간엽 줄기세포를 투여한 군과의 림프조직에서의 pro-inflammatory cytokine인 TNF-α과 IL-17f의 발현는 유의적인 차이는 없었으나, IFN-γ의 발현은 사람지방유래 중간엽 줄기세포를 투여군에서 유의성있게 대조군에 비해 증가하였다. Anti inflammatory cytokine인

TGF-β1는 대조군과 사람지방유래 중간엽 줄기세포를 투여군에 유사한 발현을 보았으며, IL-10 및 indoleamine-2,3 dioxygenase (IDO)의 발현는 사람지방유래 중간엽 줄기세포를 투여군에서 대조군에 비해 유의적으로 증가하였다.

척수실질내 염증세포의 침윤정도는 사람지방유래 중간엽 줄기세포를 투여한 군에서 대조군에 비해 완화되었다.

또한, 정맥내 투여한 사람지방유래 중간엽 줄기세포는 척수실질내에서 소수의 세포가 관찰되었고, 비장에서는 척수조직에서 비해 많은 수의 이식한 사람지방유래 중간엽줄기세포가 관찰되었다.

이상의 결과를 종합해보면, 사람지방유래 중간엽 줄기세포는 급성 자가면역성 뇌척수염을 유도한 랫트에서 임상적 완화효과를 보이며, 이는 염증매개세포인 T cell의 증식억제 및 척수조직 실질에 염증세포의 침윤억제에 의한 것으로, anti-inflammatory cytokine 인 IL-10 과 indoleamine-2,3 dioxygenase의 발현의 증가로 인한 것으로 사료된다.

이는 급성 자가면역성 뇌척수염과 같은 질병에 대해 사람지방유래 중간엽 줄기세포가 세포치료제로서 역할을 할 수 있다고 사료된다.

주요어 : 사람지방유래 중간엽 줄기세포, 자가면역성 뇌척수염, Interleukin-10, Indoleamine 2,3-dioxygenase

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그리운 것은 왜 뒤쪽에 있는지
왜 가슴 속에서 바스락 소리를 숨겨놓고 있는 것인지
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