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Original Article

The Effect of Transplanted Human Wharton's Jelly Mesenchymal Stem Cells on Matrix Metalloproteinases in Brain of Experimental Autoimmune Encephalomyelitis Mice

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Abstract

Mesenchymal stem cells (MSCs) have immunosuppressive and anti-inflammatory properties and thus offer a potentially attractive therapeutic option for autoimmune diseases such as multiple sclerosis (MS). Matrix metalloproteinases (MMPs) are a family of extracellular enzymes that play a key role in the pathogenesis of MS. It is unclear whether MSCs affect MMPs or not. In this study, we evaluated the effect of Human Wharton's Jelly Mesenchymal Stem Cells (hWJ-MSCs) on gene expression of MMPs in mice with experimental autoimmune encephalomyelitis (EAE). We isolated hWJ-MSCs based on explant culture. HWJ-MSCs were injected on days 3 and 11 after EAE induction. In the peak phase of disease (day 22) and 50 days after EAE induction, the brains were harvested and the expression of MMP-2, MMP-9, and tissue inhibitor of metalloproteinases-1 (TIMP-1) genes in brain of EAE mice was studied. HWJ-MSCs significantly decreased the expression of MMP-2 and MMP-9 genes but increased gene expression of TIMP-1 in brain of EAE mice. Our finding open new perspectives for understanding the mechanisms of MSCs in treatment of MS.

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Introduction

Multiple sclerosis (MS) is an autoimmune disorder that results in progressive demyelination through the infiltration of pathogenic T-cells into the central nervous system (CNS) [1, 2]. Matrix metalloproteinases (MMPs) are a family of zinc-containing extracellular enzymes, which can cleave extracellular matrix components and the ectodomains of several membrane proteins [3, 4]. There is evidence that MMPs play a key role in the pathogenesis of many neuroinflammatory diseases such as multiple sclerosis (MS). The production of MMPs by leukocytes helps destroy the basement membrane within the central nervous system and surrounding cerebral vessels. MMPs cause demyelination and axonal injury, promoting neuroinflammation and playing a central role in the migration of inflammatory cells into CNS [5-8]. Several members of the 25-member MMP family are increased in MS and experimental autoimmune encephalomyelitis (EAE).

Elevated levels of MMP-2, MMP-7, MMP-9, and MMP-12 have been reported in the brain and spinal cord of EAE-induced rodents (mice), as well as in cerebrospinal fluid of human MS patients [9-14]. EAE is attenuated in mice deficient for MMP-8 and MMP -9, as well as in MMP-2 and MMP-9 double-null mice [8, 15].

Mesenchymal stem cells (MSCs) are multipotent stem cells capable of self-renewal and multiple differentiations, which display neuroprotective, anti-inflammatory, and immunosuppressive properties in auto immune diseases such as MS [16-18]. Several studies have shown that adult MSCs can affect the immune T- and B-cell response. MSCs suppress cytotoxicity, cytokine secretion, and proliferation of T-cells, shifting the balance of Th1/Th2 to Th2 cells and inducing regulatory T cells (Tregs). MSCs inhibit the proliferation of B-cells and arrest their cell cycle, as well as inhibiting the production of immunoglobulin and co-stimulatory molecules of B-cells. MSCs inhibit the activation, maturation, antigen presentation, and secretion of inflammatory cytokines of dendritic cells. Furthermore, MSCs inhibit proliferation, cytotoxicity, and IFN-γ secretion by active natural killer (NK) cells [17-22]. According to the key role of MMPs in MS and EAE severity, as well as anti-inflammatory and immunosuppressive properties of MSCs, in this article, we evaluated the effect of Human Wharton's Jelly Mesenchymal Stem Cells (hWJ-MSCs) in the expression of MMPs in brain of EAE mice.

Materials and Methods

Isolation and culture of hWJ-MSCs

The collection of umbilical cords was approved by Research Ethics Committee of Baqiyatallah University of Medical Sciences, Tehran, Iran. 15 to 30 cm long pieces of human umbilical cords were collected from normal births delivered by cesarean section and vaginal delivery after full-term pregnancy following obtaining written informed

consent from mothers. Isolation of hWJ-MSCs was accomplished as previously described [23, 24]. Briefly, first the two arteries and the vein were pulled away, and the soft gel tissue was then chopped into 3-5 mm pieces, which were seeded onto the 10 cm^2 culture dish with Dulbecco's Modified Eagle Medium containing F-12 nutrient mixture (DMEM-F12; Gibco) supplemented with 10% (v/v) fetal bovine serum (FBS; Gibco), penicillin (100 U/ml), streptomycin (100 µg/ml), and amphotericin B (2.5 µg/ml) at 37°C and 5% CO_2 for 10 days. After 10 days, the pieces were removed, trypsinized with 0.025% trypsin containing 0.02% EDTA (Gibco) when the adherent cells reached 80% confluence, and passaged into a new flask. In this study, the cells in passage 3 were used.

Immunophenotype

After the third passage, the cells were trypsinized with 0.25% trypsin and labeled with the following antibodies (0.02%): FITC-conjugated mouse anti-human CD44, FITC-conjugated mouse anti-human CD34, PE-conjugated mouse anti-human CD31, FITC-conjugated mouse anti-human CD73, FITC conjugated mouse anti-human HLA-DR, FITC conjugated mouse anti-human CD117, PE-conjugated mouse anti-human CD45, PE conjugated mouse anti-human CD90, FITC conjugated mouse anti-human CD105, and FITC conjugated mouse anti-human HLA-I according to the manufacturer's recommendations for flow cytometry.

FITC-conjugated mouse IgG1 and PE-conjugated mouse IgG1 were used as isotype controls (all the antibodies were purchased from eBioscience). The cells were analyzed using FACS can flow cytometer (Becton Dickinson, San Diego, CA, USA). At least 20,000 events were recorded for each sample, and the data were analyzed using FlowJo™ software.

In vitro differentiation of hWJ-MSCs

HWJ-MSCs from passage 3 were trypsinized and plated in 4-well plates at 3×10^4 cells per well in DMEM and treated with osteogenic [10 nM dexamethasone (Sigma), 50 µg/ml ascorbic acid 2-phosphate (Sigma), and 10 mM β-glycerol phosphate (Sigma)] or adipogenic [50 µg/ml indomethacin (Sigma) and 100 nM dexamethasone (Sigma)] differentiation medium. The medium was replaced every two or three days. After 21 days, the plates were washed with PBS, fixed with 4% paraformaldehyde, and stained with Alizarin Red for osteogenic differentiation and with Oil Red for adipogenic differentiation.

Induction of EAE and treatment protocol

EAE was induced in female C57BL/6J mice when they were 6-8 weeks of age. The mice were purchased from Pasteur Institute of Iran and treated according to the guidelines of Animal Ethics Committee of Baqiyatallah University of Medical Sciences. EAE was induced by subcutaneous injection of 300 µg myelin oligodendrocyte glycoprotein (MOG) 35–55 in complete Freund's adjuvant containing 4 mg/ml of Mycobacterium tuberculosis (H37Ra) into two flanking sites of each mouse. 400 ng pertussis toxoid was intraperitoneally injected on days 0 and 2 post-immunization (34). 1×10^6 hWJ-MSCs were resuspended in 200 µl PBS (without Ca^{2+} and Mg^{2+}) and injected through the tail vein on days 3 and 11 post-immunization.

The clinical scores were graded according to a standard clinical index as follows: 0, no disease; 1, limp tail; 2, hind limb weakness; 3, paresis of one hind limb; 4, complete hind limb paralysis and 5, death [25, 26]. Control mice received HFFF-PI6 cell line [National Cell Bank of Iran (NCBI) at Pasteur Institute of Iran].

RNA isolation and cDNA synthesis

In the peak phase of disease (22 days after immunization) and day 50 of study, the brains were harvested and homogenized by a BBX24B Bullet Blender Blue homogenizer in a solution containing 0.5 mm RNAse free beads (Next Advance, Averill Park, NY, USA).

RNeasy Lipid Tissue Mini Kit (Qiagen, Valencia, CA, USA) was used to isolate RNA from the homogenized tissue. cDNA synthesis was done from 2 µg of total RNA in 75 mM KCL, 50 mM Tris-HCl (pH = 8.3), 3 mM MgCl, 300 µM dNTP, 10 mM dithiothreitol, and 2.7 mM random primers (Vivantis) in a final volume of 46 µl. The samples were heated at 65°C for 10 min and rapidly chilled on ice, and 100 U of M-MuLV reverse transcriptase was added to them (Vivantis, USA). The final mixture was incubated at 37°C for 60 min and then heated at 95°C for 5 min.

Gene expression analysis

Real-time PCR was performed in 96-well, clear optical reaction plates with Optical Adhesive Covers (Qiagen). The primers are shown in Table 1. The master reaction mixtures included the following: 6.25 ml of SYBR Green PCR Master Mix (Takara Bio), 10 pmol of primer (1 µl), 1 µl of cDNA to serve as a template, and 4.5 ml of nuclease-free water. Cycling was performed on a Qiagen thermocycler (Rotor-GeneQ, Qiagen) using SYBR Green detection and a two-step PCR protocol. Thresholds were uniformly set across all the analyzed genes with a particular reference standard to calculate ΔCT [ΔCT = CT (gene) - CT (standard)]. The β-2microglubolin expression level was evaluated as an internal control. Finally, fold changes or expression levels relative to control were considered for analysis.

Table 1. Sequences of oligonucleotide primers used for amplification in Real-Time PCR.

Primer	Sequence (5′→ 3′)
MMP-2 F	TTCCCTAAGCTCATCGCAGACT
MMP-2 R	CACGCTCTTGAGACTTTGGTTCT
MMP-9 F	CCATGCACTGGGCTTAGATCAT
MMP-9 R	CAGATACTGGATGCCGTCTATGTC
TIMP-1 F	CCAGAGCCGTCACTTTGCTT
TIMP-1 R	AGGAAAAGTAGACAGTGTTCAGGCTT
b2m F	GCTATCCAGAAAACCCCTC
b2m R	CCCGTTCTTAGCATTTG

Statistical analysis

Statistical analysis was performed using GraphPad Prism (version: 5.04). T-test and one-way ANOVA were used to evaluate the differences between groups. The relative expression of all genes of interest was evaluated as the observed Ct values, which was measured using the relative expression RT2 profiler software (http://pcrdataanalysis.sabiosciences.com/pcr/arrayanalysis.php). P values less than 0.05 were considered as statistically significant.

Results

Characterization of hWJ-MSCs

Ten days after removal of tissue fragments, fibroblast-like and single-spindle cells became detectable near the fragments. 18-20 days after initial plating, hWJ-MSCs formed colonies and reached confluency. After passage, strong proliferative activity was noted without visible alterations in growth and morphology (Fig. 1A).

The flow cytometry analysis indicated that hWJ-MSCs were positive for CD44, CD73, CD105, CD90, and HLA-I, but were negative for CD34, CD31, CD45, CD117, and HLA-DR, which showed that the cells were mesenchymal stem cells (Fig. 1D).

Osteogenic and adipogenic differentiation

For adipogenic differentiation, Oil Red O staining after 21 days in hWJ-MSCs indicated numerous lipid vacuoles (Fig. 1B). Furthermore, when the cells were induced to differentiate into osteoblasts after 21 days, massive calcium depositions were observed by Alizarin Red staining (Fig. 1C), which showed the mesodermal origin of isolated cells.

Gene expression analysis

To analyze the effect of hWJ-MSCs on the expression of MMPs genes in the brain of EAE mice, relative expression of MMP-2, MMP-9, and TIMP-1 genes was investigated using a sensitive real-time PCR method.

In the peak phase of disease (22 days after immunization), the fold changes of MMP-2 and MMP-9 genes were significantly decreased in hWJ-MSCs treated compared to HFFF-PI6 treated group ($P < 0.005$), and the fold change in the expression of TIMP-1 mRNA was significantly increased in hWJ-MSCs treated compared to HFFF-PI6 treated group ($P < 0.005$) (Fig. 2A).

In day 50 of study, the fold changes of MMP-2 and MMP-9 genes were also significantly decreased, and the fold change of TIMP-1 was significantly increased in hWJ-MSCs treated compared to HFFF-PI6 treated group ($P < 0.005$) (Fig. 2B).

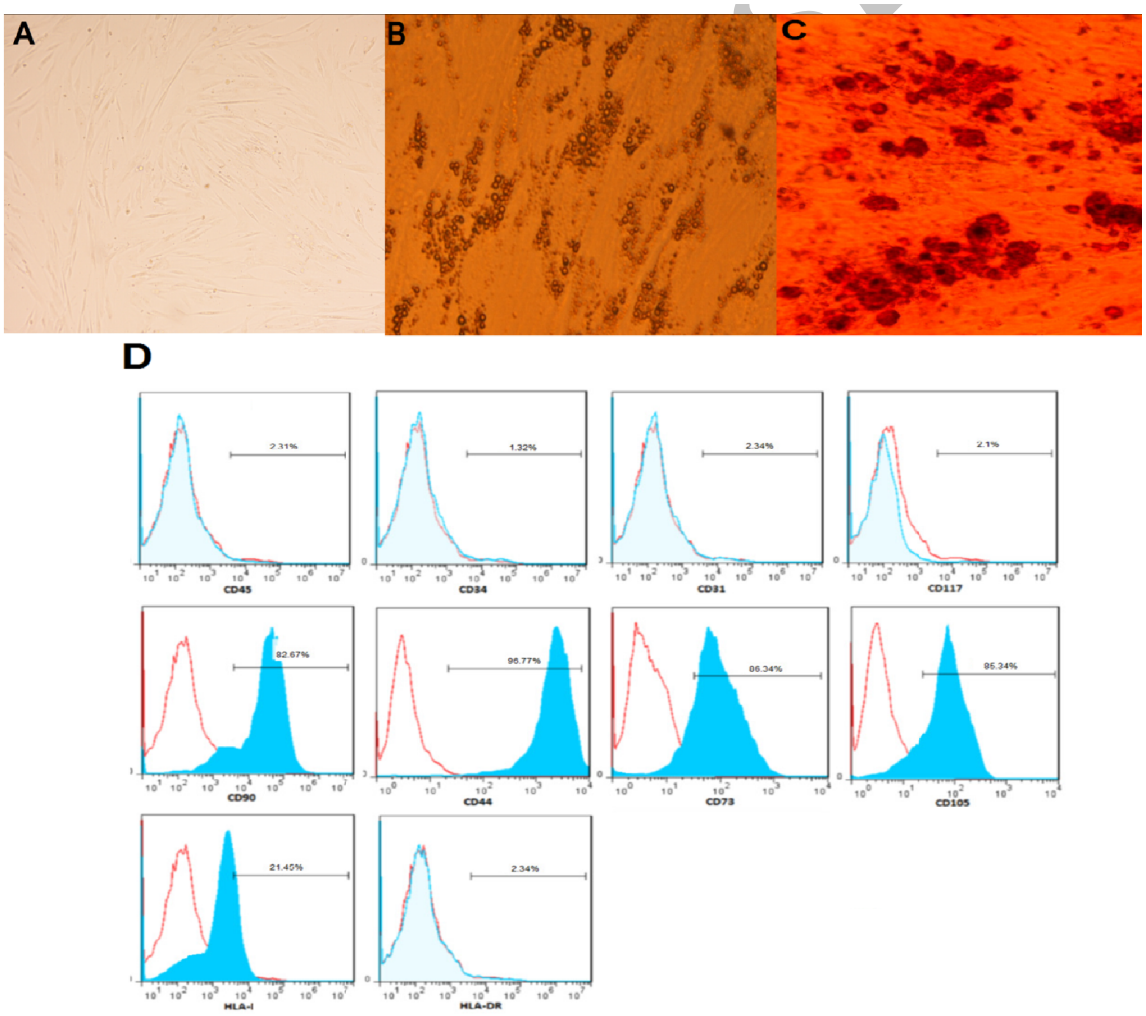

Figure 1. Isolation and characterization of hWJ-MSCs. A: HWJ-MSCs after isolation-3rd passage. B: After 21 days, adipogenic differentiation was determined by Oil Red O staining of WJ-MSCs, and intracellular lipid accumulation was stained bright red. C: Alizarin Red S staining of WJ-MSCs, and calcium deposition was stained bright orange-red in osteocytes at day 21.

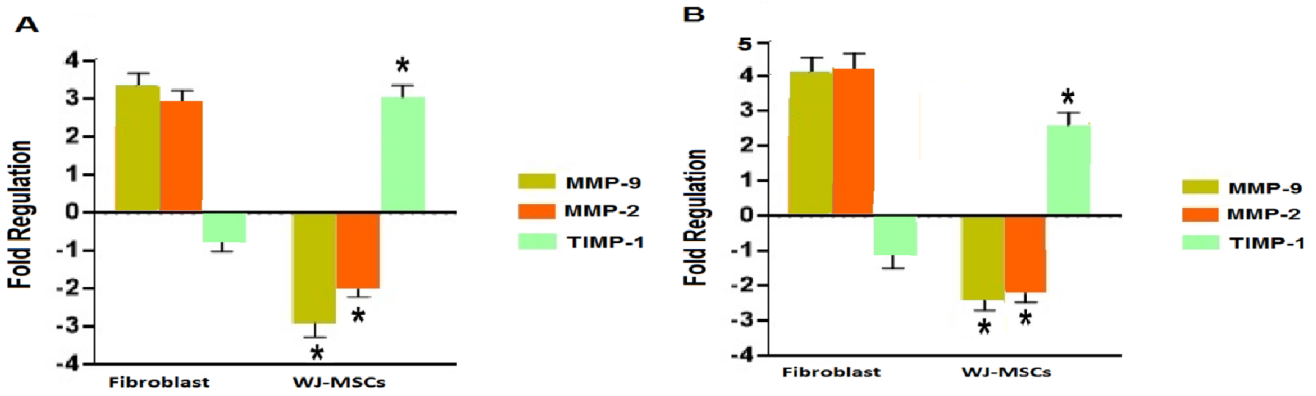

Figure 2. Effect of WJ-MSCs in the expression of MMPs gene in brain of EAE mice. A: In peak phase of disease (day 22), WJMSCs significantly decreased the expression of MMP-2 and MMP-9 in brain of EAE mice compared to HFFF-P16 treated group ($P < 0.05$). B: WJMSCs significantly decreased the expression of MMP-2 and MMP-9 in the brain of EAE mice compared to HFFF-P16 treated group in day 50 ($P < 0.05$).

Discussion

In this paper, we examined the effect of hWJ-MSCs on MMPs in EAE mice.

Mesenchymal stem cells are multipotential non-hematopoietic progenitor cells that can differentiate into multiple tissues. MSCs have immunosuppressive properties and express low levels of HLA-I but do not express costimulatory molecules such as CD80, CD86, or CD40 and are able to suppress the activation and proliferation of T- and B-lymphocytes, NK cells, and dendritic cells [21, 27-31]. Based on these properties, MSC have recently emerged as promising cellular vehicles for the treatment of autoimmune diseases such as MS.

Matrix metalloproteinases (MMPs) are endopeptidases that are dependent on calcium and zinc, which are involved in the degradation of extracellular matrix and tissue regeneration [32-34]. Recent evidence suggests that these enzymes are involved in the regulation of vital functions such as survival, angiogenesis, inflammation, and cell signaling. During inflammation, MMPs are responsible for the degradation of cellular matrix, which is necessary for the invasion of leukocytes into inflamed tissues [5]. In adult CNS, the expression of MMPs is at a low level or non-quantifiable, but the overexpression of MMPs has been reported in neurologic disorders, injuries, MS, Alzheimer's disease, viral infections, and stroke [35]. In CNS of people with MS, neuronal cells and leukocytes that infiltrate into the CNS cause increased expression of MMPs. MMPs promote nerve inflammation, damage to blood brain barrier, demyelination, and toxicity to neuronal cells. MMP 2, 7, and 9 enzymes are increased in the serum of relapsing-remitting MS patients compared with healthy subjects [36]. MS patients show the overexpression of MMP-9 mRNA in leukocytes, as well as elevated levels of MMP-9 in serum. Patients with high levels of MMP-9 and a low level of tissue inhibitor of metalloproteinase 1 (TIMP-1) have a tendency to aggravate their disease. Increased expression of MMP2, 3, 7, 9, and 14 in serum and CSF of MS patients, as well as the increased expression of MMP-3, 7, 9, 12, and 14 has been observed in mouse EAE models [37]. Migration of Th1 cells, which is involved in the pathogenesis of MS, has been further compared with Th2 cells. This is related to high levels of MMP-2 and MMP-9 in the Th1 cells [34]. These studies show the vital role of MMPs in the pathogenesis of MS. Given that the effect of MSCs on MMPs has not been so far studied in patients with MS, in this study, the expression of MMP-2, MMP-9, and TIMP-1 was evaluated in brain of EAE mice in the peak phase of disease (22 days after immunization) and day 50 of study. HWJMSCs significantly decreased the expression of MMP2 and MMP9 but significantly increased the expression of TIMP-1 in brain of EAE mice compared to HFFF-P16 treated group.

Conclusion

For first time, we have shown that hWJ-MSCs could affect MMPs and decrease their level in EAE mice. Taken together, decreased expression and function of MMPs may be a mechanism used by MSCs for treatment of MS.

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