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Research Article

Evaluating Effect of Mesenchymal Stem Cells on Expression of TLR2 and TLR4 in Mouse DCs

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Abstract

Purpose: Mesenchymal stem cells (MSCs) are multipotent cells and recent findings suggest immunomodulatory effect of them on immune cells including T cells and dendritic cells (DCs). DCs are the most potent antigen presenting cells. It seems because of immunoregulatory properties of MSCs, they can affect the maturation and differentiation of DCs. DCs express a kind of surface receptors called toll-like receptors (TLRs) and play a key role in maturation process and activation of DCs. The aim of this study was to evaluate expression of TLR2 and TLR4 on DCs after exposure to mesenchymal stem cell's supernatant in culture media containing LPS and devoid of it.

Methods: In this experimental study, MSCs and DCs were extracted from adult Balb/c mouse bone marrow and spleen, respectively. MSCs supernatant were collected 24 and 48 h after 5th passage, and in adjusted with DCs culture. Isolated DCs were co-cultured with MSCs supernatant, incubation time were 24 and 48 hours. mRNA levels of TLR2 and TLR4 were evaluated using real time PCR technique.

Results: The results demonstrated that although, expressions of these two receptors were up-regulated in culture media lacking LPS in comparison with the control group but the increase was not significant. There were no significant associations between LPS stimulated DCs with and without MSCs supernatants.

Conclusion: According to the results presented here, it appears that TLR2 and TLR4 gene expressions on the DCs are not affected by MSCs supernatant.

Introduction

Mesenchymal stem cells (MSCs) are multipotential cells isolated from various tissues such as skeletal muscle, adipose tissue, umbilical cord, amniotic fluid, peripheral blood, dental pulp, lung, and liver but its main source is bone marrow.[1-3] MSCs have self- renewal, differentiation potential, and can be used in tissue engineering.[4] MSCs express low level of MHC class I and are negative for MHC class II and co-stimulatory molecules such as CD80, CD86, and CD40. These cells have inhibitory effects on function and proliferation of immune cells such as T cells, B cells, natural killer (NK) cells, and dendritic cells (DCs).[5-8] Based on this limited immunogenic property and also according to the anti-inflammatory effects of these cells, MSCs are used for treatment of immune mediated diseases like autoimmune diseases and transplantation.[9,10]

DCs derived from hematopoietic bone marrow progenitor cells[11] and are the most potent antigen presenting cells (APCs).[12] They play a key role in directing cellular immune responses against self and foreign antigens. Immature DCs (iDCs) are found in most tissues, and constantly capture the antigens from their environment and present them to T cells. Activation and maturation of iDCs is stimulated directly by pathogens or indirectly by inflammatory cytokines. Mature DCs up-regulate MHC and co-stimulatory molecules, and migrate to lymph nodes.[2,13,14] The main DC maturation factors include lipopolysaccharide (LPS), tumor necrosis facor-α (TNF-α), methylated CpG-containing DNA, and interferon-γ (IFN-γ).[15] Such factors exert their action on DCs via stimulation of toll-like receptors (TLRs).[16]

TLRs are expressed in many antigen presenting cells such as macrophages and DCs. TLRs are fundamental molecules mediated between innate and adaptive immune responses.[17] Mammalian TLRs comprise a large family (10 members in humans and 13 in mice) that recognize wide range of pathogens.[18,19] TLRs are parts of the pattern recognizing receptors (PRRs) and recognize pathogen associated molecular patterns (PAMPs) and also damage associated molecular patterns (DAMPs) which results in production of several pro-inflammatory cytokines which participate in immune related diseases.[18,20,21] Detection of invading pathogens by DCs using TLRs is followed by pre-inflammatory cytokine production and increase antigen presentation to naïve T

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cells and ultimately sets off antigen specific immune response.[19]
Some related articles show that MSCs inhibit the up-regulation of CD1a, CD40, CD80, CD86, and HLA-DR in DCs through differentiation and can prevent rise of CD40, CD86, and CD83 expression during DC maturation.[22] MSCs supernatants have no effect on DCs differentiation, but they inhibit the up-regulation of CD83 during maturation.[23] Effects of human MSCs on the differentiation, maturation, and function of DCs derived from CD14 monocytes in vitro were also studied before.[24] The differentiation of monocyte to DC by granulocyte-macrophage colony-stimulating factor (GM-CSF) plus interleukin-4 (IL-4) can be inhibited by MSCs reversibly.[2] In addition MSCs can interfere with the development of both conventional and plasmacytoid DCs including DC function; migration, maturation and antigen presentation.[25]
Although efforts are ongoing to find out the capabilities of these cells, some studies reveal functional recovery[26-28] and it is better to move researchers toward the clinical trials.
Studying on effects of MSCs on DCs TLRs was a gap in research. So in the present study, bone marrow derived MSCs supernatant were exposed to spleen derived DCs to determine the effect of MSCs on TLRs expression of DCs in presence and absence of LPS. Expression of TLR2 and TLR4 on DCs were evaluated by real time PCR.

Material and Methods

Mesenchymal stem cells isolation

6-8 weeks old Balb/c mice were purchased from Shiraz University of Medical Sciences animal house and kept under standard conditions regarding humidity, temperature, and food. Mesenchyaml stem cells were isolated from femurs and tibias bone marrow. Briefly, isolated cells were cultured in sterile 25-cm^2 flasks that contained DMEM (Gibco, USA), 10% FBS (Sigma, USA), and 1% penicillin streptomycin antibiotics (Cinnagen, Iran). The flasks were incubated at 37°C with 5% CO_2. After 48 hours, non-adherent cells were removed and adherent cells were trypsinized and passaged.[29] In passage 5, 24 and 48 hours after trypsinization, supernatant of cells were collected. Non adherent cells were discarded after 2 days with changing the media. Characterization of MSCs was evaluated by immunocytochemistry technique for detection of fibronectin -a mesenchymal cytoskeletal marker- as previously described. The MSCs purity was more than 96% in 5th passage.[30]
Immunocytochemistry: Fibronectin, as a MSC marker, was used in order to evaluate the characterization of MSCs. The cultured cells were plated in 60-mm dishes contained cover slips coated with gelatin. The cells were washed with PBS three times, fixed with acetone for 10 min and washed with PBS again. They were treated with 0.3% Triton X-100 for 1 h, and non-specific antibody reaction was blocked with 10% normal goat serum at room temperature (RT) for 30 min. It was followed by incubation with monoclonal mouse anti-fibronectin antibody (Chemicon,UK) for 2 h (1:350 dilution), and anti-mouse FITC conjugated antibody (Chemicon,UK) for 1 h (1:150 dilution) at RT. The labeled cells were visualized using fluorescence microscope and digitally photographed (Zeiss, Axiophot,Germany).

In vitro multilineage differentiation studies

Adipogenesis and osteogenesis for -MSCs was evaluated in the appropriate induction media. The differentiation phenotype was documented using oil red O for adipocytes, and alizarin staining for osteocytes.

DCs isolation

Dendritic cells were isolated from spleens of 6-8 weeks old Balb/c mice using Nycodenz gradient medium (Axis Shields, Norway). Briefly, after removing the spleens from mice, they were digested with 1 mg/ml collagenase D (Roche, Molecular Biochemicals, Mannheim, Germany) and 0.02 mg/ml DNase (Roche, Germany) and cultured in RPMI-1640 (Sigma, USA) with 5mM EDTA. Cells were then washed and centrifuged with 300g in 4°C for 10 minutes. The pellet was re-dissolved in culture media and low density cells were depleted by density gradient on Nycodenz (D = 1.068). DCs were extracted at the interface and cultured in media containing FCS 10% (Gibco, USA) for 2 hours in incubator at 37°C with 5% CO_2. After 2 hours incubation, plates were gently washed. Non-adherent cells were discarded. Media was added to adherent dendritic cells and incubated at 37°C with 5% CO_2 for 14 h.[31] After that, DCs were collected and immediately used in the assays. DCs isolated using this method were 90% viable and purity was found to be about 95%.
All phases of the experiment were ethically approved by Shiraz University of Medical Sciences committee of ethical principles.

Flow cytometry analysis of DCs and MSCs

DCs were stained by CD11c-PE, conjugated antibodies (eBiosciences, USA). To show background fluorescence values, control staining of cells with appropriate conjugated isotypes was carried out.Also by using the following antibodies: anti-SCA-1, anti-CD45, anti-CD44 PE labeled and anti-CD34 FITC labeled antibody MSCs were stained and the purity was checked. Data of flow cytometry were analyzed by WinMDI 2.8 software (Scripps, CA, USA) and the percentage of positive cells over total cells and mean fluorescent intensity (MFI) for different markers were reported.

Experimental design

MSCs supernatant were collected after 24 and 48 hours and culturing with fresh DCs in presence and absence of LPS. After 24 and 48 hours of incubation, expression of receptors was evaluated (Table 1).

Table 1. The experimental groups. As mentioned in the table, DCs have treated with 24 and 48 hours MSCs supernatant in two incubation times (24 and 48 hours)

Samples	DC	24 hour MSCs supernatant	48 hour MSCs supernatant	LPS	Incubation Time
1	+	+	-	-	24h
2	+	+	-	-	48h
3	+	-	+	-	24h
4	+	-	+	-	48h
5	+	+	-	+	24h
6	+	+	-	+	48h
7	+	-	+	+	24h
8	+	-	+	+	48h
9	+	+	-	-	24h
10	+	+	-	-	48h
11	+	-	+	-	24h
12	+	-	+	-	48h
13	+	+	-	+	24h
14	+	+	-	+	48h
15	+	-	+	+	24h
16	+	-	+	+	48h

Primer Design

After evaluation of the eight genes as internal controls, including ACTB, GAPDH, RPL13a, PPIB, PolR2A, PRKG1, B-actin, and TBP, finally the B-actin gene was used as internal control because of its minor fluctuations during our sample collection. The primer was designed by primer blast for TLR2 (NC_000069.6), TLR4 (NC_000070.6) and β-actin (NM_001101.3) as the internal control. The thermodynamic parameter and secondary structure were determined by mfold software. The primer position in relation to exon-exon domains were evaluated by Spidey Software (www.ncbi.nim.gov/SpideyUSA) and their specificity was analyzed by BLASTn (www.ncbi.nlm.gov/BLASTnUSA).
The primer sequences for TLR2, TLR4 and β-actin were as follows:
TLR2F: 5'-TGCACGGCCACAGGCTTCAG-3'
TLR2R: 5'-GGTGGCACAGGGCACCTACG-3'
TLR4F: 5'-GGTGGCACAGGGCACCTACG-3'
TLR4R: 5'-GAAGGCTTCCACAAGAGCCGGA-3'
β-actinF 5'-ATCTACGAGGGCTATGCTCTCC-3'
β-actinR: 5'-AGCCTCGGTCAGGATCTTCAT-3'

Real time PCR

RNA was extracted from samples using RNX-PLUS$_{TM}$ (Cinnaclone, Iran) and cDNA synthesized by MMULV (Cinnaclon, Iran). Real-Time PCR technique was used for quantitative analysis of TLR2 and TLR4 mRNA expression using SYBR premix EX taq (Takara, Japan) and Step One Plus™ Real-Time PCR system (life technologies, USA). Real time PCR conditions include 95°C for 2 min as initial denaturation, 40 cycles of 95°C for 30 sec, and 64°C for 20 sec as elongation, Each assay was carried out in duplicate. β-actin was used as a housekeeping gene for normalization. Finally, expression of each target gene in comparison with reference gene was calculated using $2^{-\Delta\Delta CT}$ formula.

Statistical analysis

Data were represented in two independent experiments and presented as mean ± standard deviation (SD). The differences between groups were analyzed by one way ANOVA and Tukey test using Graph Pad Prism 5 software (Graph-Pad Software Inc, San Diego, CA). P value < 0.05 was considered significant.

Results

DCs and MSCs characterization

Characterization of MSCs was evaluated by immunocytochemistry technique for detection of fibronectin-a cytoskeletal marker-. More than 96% of cells were positive for this antigen (Figure 1).[30] The purity of DCs was evaluated by anti-CD11c-PE conjugated antibody. About 94% of isolated cells were positive for CD11c (Figure 2).

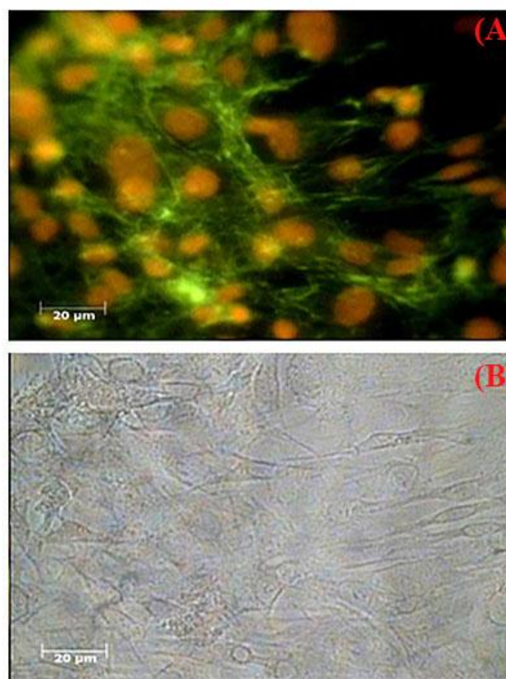

Figure 1. A photomicrograph of immunohistochemistry for fibronectin used for characterizing the MSCs at fifth passage, immunostained with anti-fibronectinAb (primary Ab) followed by incubation with FITC-conjugated secondary Ab and counterstained with ethidium bromide.B:A phase-contrast of the same image (scale bar 20 mM).

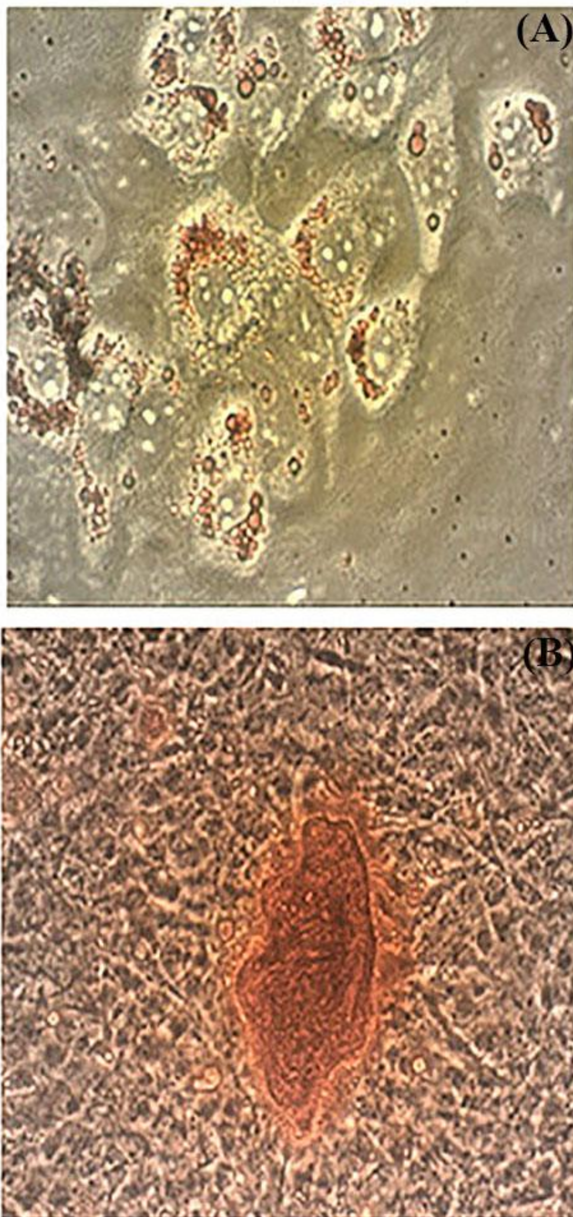

Figure 4. Differentiation potential of MSCs obtained from the bone marrow. A: Adipogenic differentiation of MSC shown by Oil red O staining of adipocytes (×100). B: Osteogenic differentiation of MSC shown by Alizarin red S (×100).

TLR2 gene expression

Expression of TLR2 on DCs was not altered after 24 and 48 hours incubation with 24 and 48 hours collected MSCs supernatant (P=0.9) (Figure 5B).

In Figure 5B, MSCs supernatant in culture media plus LPS, resulted an increase of TLR2 expression in all of the groups nearly same than LPS and culture (control group) only but the data statistically were not significant (P= 0.6).

After comparing samples containing LPS and without LPS we found that complex of LPS and MSCs supernatant in culture media, have no significantly effects on TLR2 expression (P> 0.05).

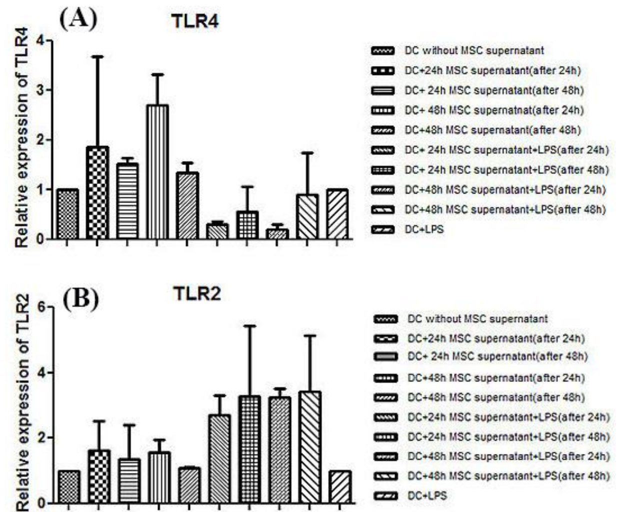

Figure 5. A: Comparison between expressions of TLR4 in all conditions with control group showed an increase but not statistically significant, and expression of TLR4 gene in all groups in presence of LPS in comparison with control group was decrease but it was not significant statistically. Experiments were carried out in duplicate and repeated 3 times.
B. Expression of TLR2 gene in all groups after adding MSCs to the culture in comparing to DC was increased but data was not significant, and TLR2 gene expression in presence of LPS and in comparison to control group was shown and non-significant increase in gene expression was shown. Assays were carried out in duplicate for 3 times.

Discussion

Mesenchymal stem cells, are immunogenic and immunomodulator cells which can affect various components of the immune system.[32] MSCs in *in vitro* condition express a small amount of MHC I and are negative for MHC II, CD40, and CD86 and, therefore, is unable to stimulate immune reaction in the host.[33] It has been documented that MSCs play their immunomodulatory functions via cell contact or secretion of certain factors.[34] Previous investigations demonstrated that DCs are the most cells which are under immunomodulatory functions of MSCs.[35,36] Toll like receptors (TLRs) have been shown to play an essential role in inducing the immune activation program in DCs.[37,38] Previous studies have shown that IL-6 and PGE-2 can effect DCs maturation.[10,39] These soluble factors are secreted by MSCs and interactions between DCs and MSCs are usually mediated by these factors.[12] IL-6 is involved in MSC-induced immunomodulation by inhibition of DC differentiation.[12] However it is not the core mechanism and is mostly involved in differentiation of monocytes to DCs.[40] PGE-2 inhibits the DC maturation in culture media containing DCs and MSCs and has a stronger effect than IL-6.[41] As immature DCs become mature, they lose expression for all TLRs except TLR1/TLR6[42] so TLR2 and TLR4 should highly expressed on imDCs in contrast to mature one. According to this finding, we supposed that MSCs supernatant inhibit DC maturation and can increase TLR2 and TLR4 expression.

Results of this study was shown that, although, MSCs supernatant in absence of LPS by increasing incubation time from 24 to 48 hours lead to decline in TLR4 and TLR2 expression but data were statistically not significant. Therefore, according to the results it appears that MSC`s supernatants are unable to induce DC maturation. According to the fact that PGE-2 is a factor which is produced by MSCs, hence, it seems that MSCs suppress DC maturation via secretion of PGE-2. The results revealed that after increasing incubation time to 48 hours, TLR2 and TLR4 expression were not changed and this approve our conclusion and confirm the inhibitory effects of MSC`s supernatant on the maturation of DCs.

Previous studies have been shown that LPS can cause DCs maturation[43] and expression levels of TLR are reported to decrease with the maturation of DCs.[42,44] Although the results were not significant, but an increased expression of TLR4 in the DCs incubated with LPS in comparison to DCs incubated with LPS and MSC`s supernatants were demonstrated. In contrast with TLR4, TLR2 expression were relatively decreased in the DCs incubated with LPS (the results were not significant) in comparison to DCs incubated with LPS and MSC`s supernatants. Previous studies demonstrated that TLR2, but not TLR4, is a pathogen recognition receptor which may play as an anti-inflammatory factor in some situations.[21] Therefore, according to the results presented in this study it appears that LPS may lead to up and down-regulation of TLR2 and TLR4, respectively, and in the DCs and MSC`s supernatant may affect increased expression of TLR4 in the treated DCs. Based on the fact that the study have not statistical significant, hence, it may propose that future studies will be needed to learn more about MSCs, DCs and TLRs.

Conclusion

In summary, our data confirmed the inhibitory effects of MSCs supernatant on the maturation of DCs and also suggested that MSCs supernatant might be modulate the expression of TLR4 and eventually suppress immune system. Although all aspects of MSCs modulatory mechanisms on DCs still remain unclear. Since, TLR activation promote capacity of DCs to start immunological reactions and inducing T cell responses, so working on other TLRs seems beneficial for treating autoimmune and immune mediated diseases.

Acknowledgments

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Ethical Issues

Not applicable.

Conflict of Interest

Authors declare no conflict of interest in this study.

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