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Chemically primed bone-marrow derived mesenchymal stem cells show enhanced expression of chemokine receptors contributed to their migration capability

Hamid Reza Bidkhori [1, 2], Naghmeh Ahmadiankia [3], Maryam Moghaddam Matin[1, 2], Asieh Heirani-tabasi [2], Moein Farshchian[1, 4], Hojjat Naderi-meshkin [2], Mina Shahriyari [2], Mahtab Dastpak [1, 2], Ahmad Reza Bahrami [1, 2, 5]*

[1] Department of Biology, Faculty of Sciences, Ferdowsi University of Mashhad, Mashhad, Iran
[2] Stem Cells and Regenerative Medicine Research Department, ACECR-Khorasan Razavi Branch, Mashhad, Iran
[3] Shahroud University of Medical Sciences, Shahroud, Iran
[4] Molecular Medicine Research Department, ACECR-Khorasan Razavi Branch, Mashhad, Iran
[5] Cell and Molecular Biotechnology Research group, Institute of Biotechnology, Ferdowsi University of Mashhad, Mashhad, Iran

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ABSTRACT

Objective(s): The limited homing potential of bone-marrow-derived mesenchymal stem cells (BM-MSC) is the key obstacle in MSC-based therapy. It is believed that chemokines and chemokine receptor interactions play key roles in cellular processes associated with migration. Meanwhile, MSCs express a low level of distinct chemokine receptors and they even lose these receptors on their surface after a few passages which influence their therapeutic applications negatively. This study investigated whether treatment of BM-MSCs with hypoxia-mimicking agents would increase expression of some chemokine receptors and cell migration.

Materials and Methods: BM-MSCs were treated at passage 2 for our gene expression profiling. All qPCR experiments were performed by SYBR Green method in CFX-96 Bio-Rad Real-Time PCR. The Boyden chamber assay was utilized to investigate BM-MSC homing.

Results: Possible approaches to increasing the expression level of chemokine receptors by different hypoxia-mimicking agents such as valproic acid (VPA), $CoCl_2$, and desferrioxamine (DFX) are described. Results show DFX efficiently up-regulate the *CXCR7* and *CXCR4* gene expression while VPA increase only the *CXCR7* gene expression and no significant change in expression level of *CXCR4* and the *CXCR7* gene was detectable by $CoCl_2$ treatment. Chemotaxis assay results show that pre-treatment with DFX, VPA, and $Cocl_2$ enhances significantly the migration ability of BM-MSCs compared with the untreated control group and DFX treatment accelerates MSCs homing significantly with a higher rate than VPA and $Cocl_2$ treatments.

***Conclusion*:** Our data supports the notion that pretreatment of MSC with VPA and DFX improves the efficiency of MSC therapy by triggering homing regulatory signaling pathways.

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Introduction

Bone marrow-mesenchymal stromal cells (BM-MSCs) have the potential to differentiate and/or reprogram into many cell lineages, secrete a variety of immune modulatory cytokines, and include stable genetic background, which makes them among the best candidates for application in stem cell therapy (1). In spite of low expression rate of surface cytokine receptors in BM-MSCs, a large number of them can be found in intracellular space and the expression rate of surface chemokine receptors is even significantly decreased during *in vitro* culture of MSCs for more than two passages (2-6). This makes it necessary to look for appropriate approaches to improve the homing capacity of the cultured cells and enhance retention of the implanted MSCs leading to better efficacy of the cell-based therapeutic practices (7).

Chemical treatment is a preferable strategy for enhancing expression of the chemokine receptors, especially if such chemicals are used as components of the approved drugs for different purposes (8).

Desferrioxamine (DFX) is a metal-chelating drug often used in iron accumulation diseases. DFX may

**Corresponding author:* Ahmad Reza Bahrami. Department of Biology, Faculty of Sciences, Ferdowsi University of Mashhad, Mashhad, Iran. email: ar-bahrami@um.ac.ir

induce hypoxic condition by stabilizing hypoxia-inducible factor-1 alpha (HIF-1a) protein (9). Recent studies show the effects of $CoCl_2$ as an HIF-1a activation-mimicking agent on MSCs (10), but there isn't any comprehensive cytokine receptor expression profiling after treatment of BM-MSCs with $CoCl_2$. VPA (2-propylpentanoic acid) is an FDA-approved anticonvulsant and mood-stabilizing drug in some neurological disorders (11). It has been reported that VPA increased acetylated histone-H3 levels of CXCR4 promoter in rat MSC (8). In the present study, we found for the first time, the effects of hypoxia mimicking agents on cytokine expression in BM-MSC and our results suggest DFX and VPA, by recruiting special signaling pathway, promote the expression rate of the cytokine receptors and would make them applicable as a therapeutic choice in MSC transplantation.

Materials and Methods

Bone marrow cell preparation and BM-MSC characterization

We enrolled patients who on physician's advice were to undergo bone marrow aspiration and had no history of prior chemotherapy or radiotherapy, after informed consent and in accordance with the ethical standards of the local ethical committee. Patient specimens that revealed abnormal pathological evaluation were excluded from the study. 5 ml of human bone marrow aspirates, taken from the iliac crest of normal donors, were diluted 1:1 with phosphate buffered saline and layered over an equal volume of Lympholyte cell separation solution (Cederlane, Canada). After centrifugation at 1500 g for 20 min, the mononuclear cells (MNCs) were recovered from the gradient interface and washed with PBS. MNCs or nonfractionated bone marrow cells were suspended in Dulbecco's modified Eagle's medium containing 1 g/l of glucose (DMEM-LG; GIBCO) supplemented with 10% fetal bovine serum (FBS; GIBCO), 100 U/ml penicillin, and 100 µg/ml streptomycin. All cells were plated in 10 ml of medium in a culture flask (tissue culture flask; orange).

BM-MSC differentiation assays

For osteogenic induction, cultures were treated with 50 mg/mL ascorbate-2 phosphate, 100 nmol/L dexamethasone (Sigma, Munich, Germany), and 10 mmol/L b-glycerophosphate (Sigma) for a period of 3 weeks (6). After washing and fixation, cells were incubated with 0.1% (wt/vol) Alizarin for detection of calcium contained structures. The adipogenic differentiation was performed based on da Silva Meirelles protocol (12) as we utilized previously (6, 13); adipogenesis potential of cells was detected after treating with 50 mg/ml ascorbate- 2-phosphate, 100 nmol/l dexamethasone, and 50 mg/ml indomethacin (Sigma) for 3 weeks and Oil red O (Sigma) staining for 20 min.

FACS analysis

For evaluation of cell surface markers of cultured BM-MSCs, 1 $\times 10^{6}$ cells at passage 4 were resuspended in 100 µl cold phosphate buffer saline (PBS), containing 5% FBS and after 1 hr incubation with respective antibodies or isotype-matched control, data was obtained using the flowcytometry instrument (BD Accuri™ C6). The antibodies sets we applied for our FACS studies were: mouse anti-CD44 polyclonal antibody, rabbit anti-CD34 polyclonal antibody (all from antibodies-online, Aachen, Germany), mouse anti-CD90 monoclonal antibody, rabbit anti-CD11b polyclonal antibody, mouse anti-CD73 monoclonal antibody (all from Novus Biologicals, Littleton, Colorado, USA), rabbit anti-CD105 polyclonal antibody, and rabbit anti-CD45 polyclonal antibody (all from Bioss Inc., Woburn, MA, USA).

Treatment of cells with drugs

MSCs were treated with various hypoxia mimicking agents for 24 hr. An incubation time of 24 hr was selected on the basis of our preliminary experiments showing that expression of the CXCR4 increases in a time-dependent manner after treatment with VPA, DFX, and $CoCl_2$, reaching a maximum between 16 and 24 hr. The MSCs were then washed extensively with 1x PBS, dissociated from T75 cell culture flasks by Accutase (Life technologies, USA) for 5 min at 37 ˚C, centrifuged (400 g, 6 min) and immediately homogenized in TriPure reagent (Roche Diagnostics, Germany) for extraction of the total RNA.

Total RNA isolation and reverse transcription

Total RNA was isolated using a commercially available reagent TriPure (Roche Diagnostics, Germany) following the manufacturer's instructions. Total RNA was treated with 1 units of DNase I enzyme (Thermo Fisher Scientific, USA), in order to avoid amplification of contaminating genomic DNA. 1000 ng of the total RNA was added to 1 µl of oligo-dT primer (100 µM) and incubated at 65˚C for 5 min. Samples were chilled on ice, and 10 µl of RT mix components was added following the manufacturer's instructions (Thermo Fisher Scientific, USA). The samples were then incubated at 42˚C for 60 min followed by an enzyme inactivation period for 5 min at 70˚C.

RT-PCR analysis

To quantify expression of the chemokine receptor genes, real-time PCR was performed using SYBR Green Master Mix (Parstoos, Iran) in the thermal cycler system (CFX-96, Biorad, USA) following the manufacturer's protocol. The forward and reverse

Table 1. Primer sequences used for quantitative RT-PCR

mRNA targets	Oligonucleotides(5'→3')	Product size (bp)
β-actin	F: GCTCAGGAGGAGCAAT R: GGCATCCACGAAACTAC	187
CXCR4	F: ATCCCTGCCCTCCTGCTGACTATTC R: GAGGGCCTTGCGCTTCTGGTG	231
CXCR7	F: AGTTGTTGCAAAGTGCTCAG R: TCTGAGTAGTCGAAGAGATGC	132

primers used for quantitative amplification of the chemokine receptor mRNAs (*CXCR4* and *CXCR7*) and those used for amplification of *β-actin* are shown in Table 1.

Migration assay

BM-MSC transwell migration assay was assessed in a 24-well plate Boyden chamber (Corning Costar Corporation, Cambridge, MA) (14) by culturing of BM-MSC suspensions into the top compartments with serum-free DMEM containing 0.5% BSA at a concentration of 25×10^3 cells/ml in 100 µl. 100 ng/ml SDF-1α was loaded as an attractive agent to the bottom compartment in a total volume of 0.6 ml, the non-migrated cells were completely cleaned from the top surface of the filters after 18 hr incubation at 37 °C and 5% CO_2, and migrated adhering cells on the membrane counted after DAPI staining. AMD3100, a CXCR4-blocking agent (15), was used at a concentration of 10 µg/ml for 30 min at room temperature for inhibition activity of SDF-1α induced migration before our transwell assay. Each experiment was performed twice in triplicate wells. Five fields were randomly selected and counted from each well and the average numbers of cells per field were considered for statistical analysis. Statistical significance was defined as *P*-values ≤0.05.

Statistical analysis

Statistical analysis was performed using the SPSS 19.0 statistical package (SPSS, Chicago, IL, USA). All data were expressed as mean ± SD and analyzed using t-test. *P*-values <0.05 or <0.01 were considered statistically significant.

Results

Characterization of the isolated cells

Authentication of bone-marrow-derived mesenchymal stem cells with the other hematopoietic cells with common niches was confirmed based on surface antigen characterization and evaluation of their differentiation capacity. A high percent of the cell population with CD44, CD105, CD73, and CD90 which are negative for CD45, CD11b, and CD34 markers (Figure 2) strongly confirmed BM-MSC nature of isolated cells. Also, identification of the differentiated cells was detected by Alizarin Red S (Figure 1a) and Oil Red O (Figure 1b) staining, to visualize calcium precipitates of osteocytes and accumulation of lipid vacuoles in differentiated adipocytes, respectively.

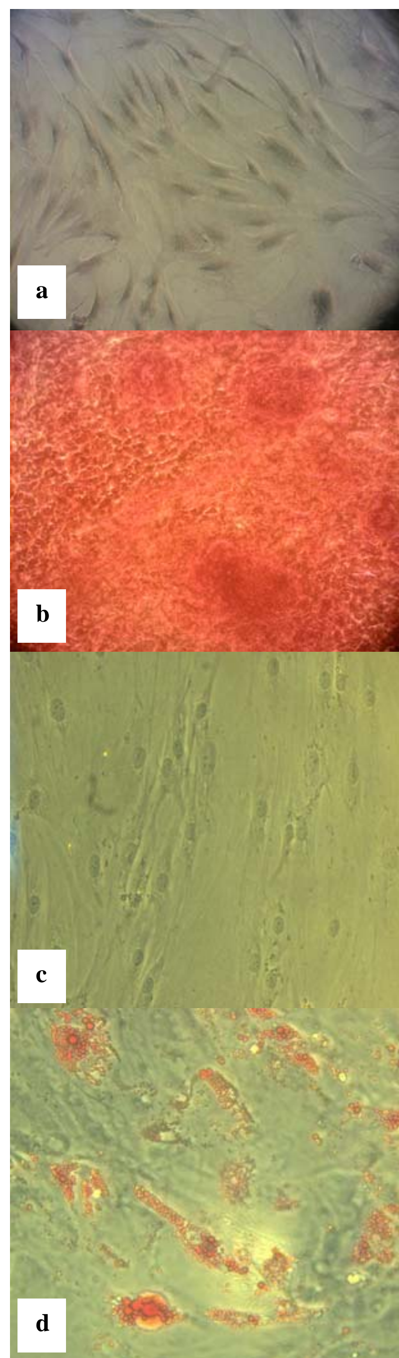

Figure 1. Osteogenic and adipogenic characterization of isolated BM-MSC. BM-MSC cells were grown in defined conditioned medium and stained with Alizarin Red (a, b) and Oil Red O (c, d). Panels a and c represent negative controls for staining

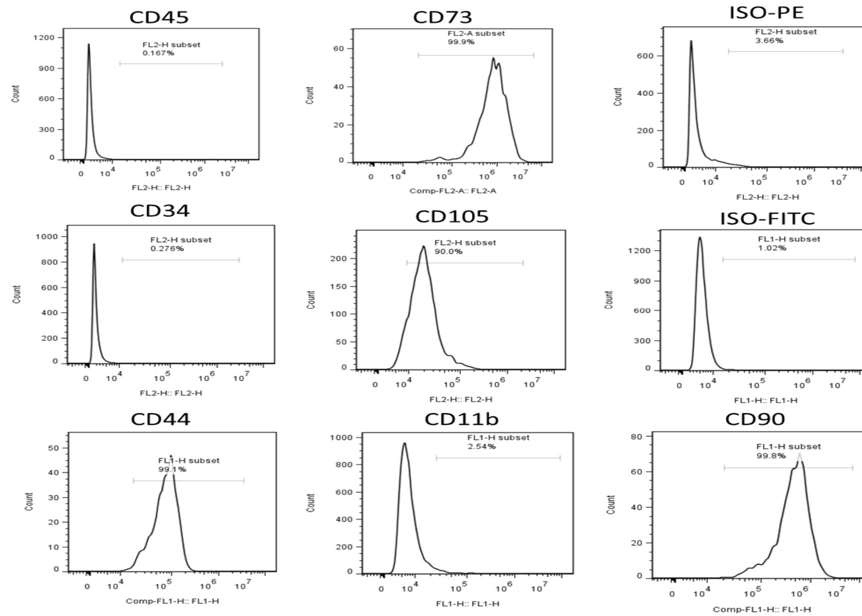

Figure 2. Phenotype characterization of BM-MSCs. Flowcytometry analysis showed that almost all cultured BM-derived MSCs expressed CD73(99.9%), CD105(90.0%), CD90(99.8%), and CD44(99.1%), whereas a small portion of them expressed CD34(0.27%), CD45(0.16%), and CD11b(2.54%)

Upregulation of CXCR4 and CXCR7 following DFX and VPA treatment

To evaluate the effects of small molecules, hBM-MSCs were incubated with 2.5 mM, 100 μM and 120 μM of VPA, $CoCl_2$, and DFX, respectively for 24 hr. We evaluated *CXCR4* and *CXCR7* mRNA expression levels in the treated samples, and compared these with the expression levels in the untreated BM-MSC at passage 2, by qRT-PCR. As shown in Figure 2, DFX efficiently upregulates *CXCR7* and *CXCR4* gene expression and VPA only increases *CXCR7* expression (P-value<0.05). $CoCl_2$ did not show any significant effect on expression of these factors. Here, we show that short-term exposure of the BM-MSCs to VPA increases only *CXCR7* gene expression, without any significant effect on the *CXCR4* expression level. Our results are consistent with the findings of others (8, 16) who reported upregulation of the *CXCR4 gene* following VPA treatment and non-effectiveness of $CoCl_2$ is in line with another study (17), which showed the induction of neuronal differentiation in human mesenchymal stem cells following $CoCl_2$ treatment. The observations do not exclude the possibility that $CoCl_2$ might be considered as a hypoxia-mimicking agent and change the expression portrait of other cytokine receptors.

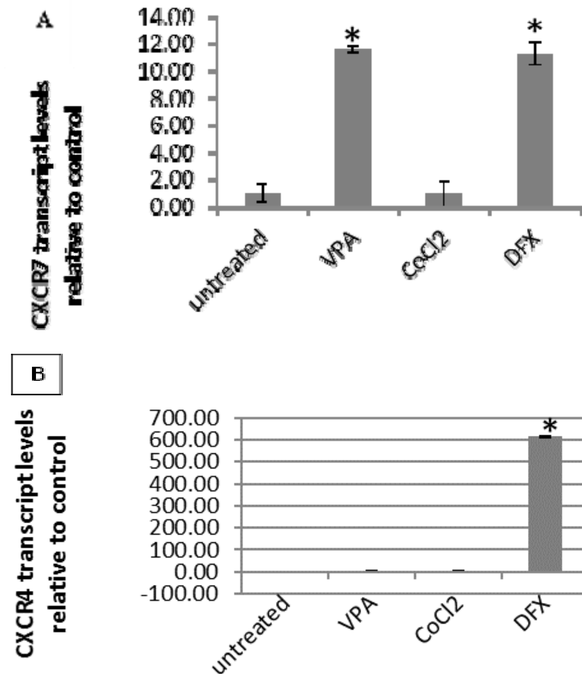

Figure 3. Effects of VPA, $CoCl_2$, and DFX on the gene expression of chemokine receptors. BM-MSCs were treated with VPA, $CoCl_2$, and DFX for 24 hr, and then subjected to real-time PCR analysis for determination of alterations in gene expression of selected chemokine receptors including CXCR7 (A) and CXCR4 (B). Data are represented as mean±SD from three independent experiments. Results were statistically evaluated by using one-way ANOVA.* P-value<0.05 was considered significant as compared with control

Enhanced chemotaxis assay

To determine whether pre-treatment of BM-MSCs with DFX, VPA, and $CoCl_2$ influences their homing,

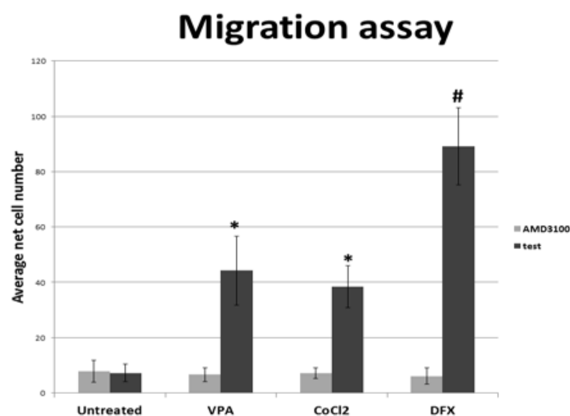

Figure 4. Transwell migration assay: # denotes significant difference relative to untreated control, VPA, and $CoCl_2$ pre-treatment. * denotes significant difference relative to untreated control

transwell migration assay (8 µm pores size, Costar, corning, MA, USA) was performed. Bone-marrow-derived MSCs with different pre-treatments were left to migrate from upper compartment towards SDF1 in the lower compartment. The migration rate of MSCs, treated with DFX, VPA, and $CoCl_2$, was significantly higher than that of the untreated control group (Figure 4). To ensure the role of SDF1/CXCR4 axis, a CXCR4 antagonist, Plerixafor also known as AMD3100, was used (18). This blockage clearly reduced the migration rate of BM-MSCs, as migration index was significantly higher in the group treated with DFX in comparison to two other hypoxia mimicking agents.

Discussion

MSCs are known as a suitable cell source that secrete several anti-inflammatory, angiogenic, and antifibrotic factors and induce immunomodulation without significant activation of the immune response. Thus, MSCs induce regeneration in the surrounding tissues and cells, which is an important beneficial effect in cell therapy (19). Systemic administration of stem cells is generally preferred to local injection due to being less invasive (20). One critical barrier to effective MSC therapy is their insufficient homing capability to tissues of interest, especially when MSCs are infused through the vascular route (21). Many factors contribute to the inefficient migration of these cells, among which low surface receptors' level is of high importance (2, 19). Some studies have highlighted that chemokine/-chemokine receptor interactions influence stem cell recruitment to the desired target, but to the best of our knowledge none of them have investigated whether chemical pretreatment of MSCs with hypoxia mimicking agents could enhance chemokine receptors expression. Both receptors of the chemokine CXCL12 (SDF1), CXCR4, and CXCR7 play a different but pivotal role in stem cell homing process. CXCR4 is required for the chemotactic activity of CXCL12 and recruitment of stem cells, whereas CXCR7 is responsible for the endothelial level (22).

In one study, it was demonstrated that enhancing of MSC homing to the pancreas, after treatment with hypoxic conditions of DFX (23), overexpression of chemokine receptors (CXCR4 and CCR2) and matrix metalloproteinase (MMP-3 and MMP-9) were elicited as a critical subsequent effect of pretreatment. Given the fact that CXCR7 plays a critical role in the CXCL12/CXCR4-mediated migration of BM-MSCs and tumoral cells (24); this could be an attractive strategy for homing the cells in cell therapies. Another study suggested that VPA has a diverse effect on *CXCR4* expression, depending on the kind of the cells being used in the investigation and cell maturation status (25).

Other reports have shown that expression of diverse factors including ANG, BDNF, ECGF1, bFGF-2, GDNF, HGF, IGF-1, PIGF, TGF-b, and Pix are increased following VPA treatment of hBM-MSCs (26). Another report (27) showed that valproic acid induces neuronal differentiation by activation of neuron progenitor genes in hBM-MSC.

Conclusion

Our results support the notion that pretreatment of BM-MSC with chemical small molecules like VPA and DFX may help to maintain the surface cytokine receptors through *CXCR7* and *CXCR4* overexpression, and produce more reliable cells for therapeutic applications.

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