Original Article
Effect of ex vivo culture density on CXCR7 expression in human mesenchymal stem cells

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Abstract: The ability of Mesenchymal stem cells (MSCs) to migrate into host tissues is crucial to their potential use in cell therapies. Here, we evaluated whether ex vivo culture conditions affect the expression of C-X-C chemokine receptor type 7 (CXCR7), which plays pivotal roles in the survival and migration of MSCs to injured tissues, in MSCs. CXCR7 was up-regulated in BM-MSCs harvested at a high density (~90% confluent) in comparison to BM-MSCs harvested at a low density (~50% confluent). Furthermore, following their injection, high-density MSCs expressing CXCR7 were found in small intestinal tissues of graft-versus-host disease mice. In addition, MSCs derived from three different umbilical cord blood samples highly expressed CXCR7 in high-density culture. Collectively, culture conditions such as cell confluency at harvest are important for CXCR7 expression in MSCs; therefore, the results of this study may provide useful guidelines for the harvest of MSCs that can migrate to injured tissues.

Keywords: Mesenchymal stem cells, cell density, CXCR7, migration, injured tissues

Introduction

Human mesenchymal stem cells (MSCs) have become therapeutically important agents [1-3] because of their multilineage potential [4], immuno-modulatory properties [5], and ability to localize specifically to injury sites [6]. MSCs are readily isolated from tissues, can be expanded in culture, and have a low tumorigenic potential. In addition, these cells tend to home to sites of tissue growth and repair, and to enhance tissue regeneration. Indeed, many MSC transplantation studies have shown beneficial effects in the treatment of damaged tissues. However, most studies report a very low degree of MSC engraftment in naive adult animals [7] and the poor retention of implanted MSCs [6, 8].

MSCs express a wide variety of cytokines, chemokines, growth factors and their receptors that are important for cell migration, homing, and immunomodulation, following reconstitution of injured tissues [9-13]. Based on their functional effects, the difference in secretion of these molecules by MSCs might have a critical effect on the results of specific applications of cell therapies. Several chemokines and their receptors are important factors that contribute to the regulation of cell migration [14]. Recently, it was shown that chemokine receptors including C-X-C chemokine receptor type 7 (CXCR7) and C-X-C chemokine receptor type 4 (CXCR4) and their ligand stromal cell-derived factor-1 (SDF-1), also known as CXCL-12, are essential components involved in the migration of cells into inflamed sites [15-19]. Thus, several attempts are currently being made to enhance the engraftment of stem/progenitor cells in vivo by increasing cell migration mediated by the SDF-1/CXCR7 or SDF-1/CXCR4 axis [20-22]. Furthermore, several studies showed that MSCs migrate in response to SDF-1 [15, 16, 23, 24], implying that upregulating CXCR7 or CXCR4 could be a useful method to enhance the migration of MSCs into damaged tissues.

Our previous report showed that the cell culture density modifies the gene expression patterns in MSCs [25]. Genes that are highly expressed
CXCR7 expression in MSCs according to cell density

in MSCs harvested at a high density are linked to functional gene expression in MSCs. Based on this, we investigated whether cell culture density affects CXCR7 expression in MSCs. The results of this study may provide useful guidelines for the collection of appropriate MSCs that migrate to injured tissues.

Materials and methods

Isolation and culture of human MSCs

The Institutional Review Board of Samsung Medical Center approved this study (IRB No. 2011-10-134), and all samples (iliac crest bone marrow (BM) aspirates) were obtained from healthy adult volunteers with informed consent. Mononuclear cells were isolated from BM aspirates or umbilical cord blood (CB) of newborns using density gradient centrifugation (Histopaque-1077; Sigma-Aldrich, St. Louis, MO). Cells were plated at a density of $3 \times 10^5$ cells/cm$^2$ in low-glucose Dulbecco’s Modified Eagle’s Medium (Invitrogen-Gibco, Rockville, MD) containing 10% fetal bovine serum (FBS; Invitrogen-Gibco) and 100 U/mL penicillin/streptomycin (Invitrogen-Gibco). The cells were incubated in a humidified atmosphere at 37°C with 5% CO$_2$, and the medium was changed every 3-4 days until adherent fibroblast-like cells reached ~70% confluency.

Phase contrast microscopy and cell counting

Viable BM-derived MSCs (BM-MSCs) at passage 2 and CB-derived MSCs (CB-MSCs) at passage 6 were used throughout these studies. The morphological appearance of MSCs was observed using a phase contrast microscope (Olympus CK40, Melville, NY). Cells plated at an initial density of 200 or 5,000 cells/cm$^2$ were incubated for 7 days in a humidified atmosphere at 37°C with 5% CO$_2$, and the medium was changed every 3-4 days until adherent fibroblast-like cells reached 70% confluency.

Characterization of BM-MSCs by immunophenotype analysis

Antibodies against the human antigens CD14, CD34, CD45, CD73, CD90, CD105, CD166, and HLA-DR were purchased from BD Biosciences (San Jose, CA). A total of $5 \times 10^5$ cells were resuspended in 0.2 mL of phosphate-buffered saline (PBS; Biowest, Nuaille, France) and incubated with fluorescein isothiocyanate- or phycoerythrin-conjugated antibodies for 30 min at room temperature. The fluorescence intensity of cells was evaluated on a BD Biosciences FACSVersa flow cytometer and data were analyzed using BD FACSuite software (BD Biosciences).

Immunoblotting

MSCs were washed with cold PBS and lysed in 300 μL of cold RIPA buffer (50 mM Tris-HCl, pH 7.5, containing 1% Triton X-100, 150 mM NaCl, 0.1% sodium deoxycholate, and a protease inhibitor cocktail (Thermo Fisher Scientific, Rockford, IL). Cell lysates were centrifuged at 3,000 × g for 15 min at 4°C. The supernatant was collected, and protein concentrations were analyzed using a bicinchoninic acid protein assay kit (Thermo Fisher Scientific). For electrophoresis, proteins (50 μg) were dissolved in sample buffer (60 mM Tris-HCl, pH 6.8, containing 14.4 mM β-mercaptoethanol, 25% glycerol, 2% SDS, and 0.1% bromophenol blue), boiled for 5 min, and separated on a 10% SDS reducing gel. Separated proteins were transferred onto polyvinylidene difluoride membranes (GE Healthcare, Buckinghamshire, UK) using a trans-blot system (Invitrogen-Gibco). Blots were blocked for 1 h at room temperature in Tris-buffered saline (TBS) (10 mM Tris-HCl, pH 7.5, supplemented with 150 mM NaCl) containing 5% non-fat dry milk (BD Biosciences), washed three times with TBS, and incubated at 4°C overnight with primary antibodies prepared in TBST (TBS supplemented with 0.01% Tween 20) containing 3% non-fat dry milk. On the next day, blots were washed three times with TBST and incubated for 1 h with secondary antibodies prepared in TBST containing 3% non-fat dry milk at room temperature. After washing three times with TBST, proteins were visualized with an enhanced chemiluminescence detection system (GE Healthcare).

RNA isolation and microarray analysis

Total cellular RNA was extracted using TRIzol (Invitrogen-Gibco) and purified using an RNeasy column (Qiagen, Valencia, CA). RNA quality was ascertained using denaturing gel electrophoresis, the OD 260/280 ratio, and analysis on an
CXCR7 expression in MSCs according to cell density

Figure 1. Characteristics of BM-MSCs. A. Morphological appearance of BM-MSCs derived from two donors (D1 and D2). B. The immunophenotype of BM-MSCs derived from D1 and D2 was analyzed by flow cytometry. The expression of surface antigens was plotted against appropriate IgG isotype controls (gray histogram). MSCs used for the analyses were positive for CD73, CD90, CD105, and CD166, and negative for CD14, CD34, CD45, and HLA-DR (clear histogram). C. The expression levels of CXCR4 and CXCR7 in BM-MSCs. Human myeloid leukemia HL-60 and THP-1 cells were used as a positive or negative control.

Quantitative real-time PCR (qRT-PCR) analysis

cDNA was produced using the Superscript™ RT-PCR System (Invitrogen-Gibco) according to the manufacturer’s recommendations for oligo(dT)$_2$-primed cDNA synthesis. qRT-PCR was performed in 384-well microtiter plates using a CXCR7 (Hs00604567_m1)-specific TaqMan® probe and primer sets (Assays-on-Demand, Applied Biosystems, Foster City, CA) and an ABI PRISM 7900HT Sequence Detection System (Applied Biosystems). Template cDNA was added to the reaction mixture, and amplifications were initiated with a 10 min template denaturation step at 95°C, followed by 40 cycles at 95°C for 15 sec and 60°C for 1 min. All samples were amplified in triplicate. Data were analyzed with Sequence Detector software (Applied Biosystems).

Immunocytochemistry

MSCs (200 and 5000 cells/cm$^2$) were plated on individual coverslips and cultured for 2 or 7 days. On Day 2 or 7, cells were placed into fixative solution (4% formaldehyde prepared in PBS) for 30 min at room temperature in the dark and washed twice with PBS. To detect internally expressed molecules, cells were permeabilized with 0.25% Triton X-100 prepared in PBS for 5 min at room temperature in the dark. Cells were washed twice, and blocking solution (5% FBS prepared in PBS) was added and incubated with the cells for 1 h at room temperature. After another washing step, an antibody against CXCR7 (1:100), purchased from Santa Cruz Biotechnology (Santa Cruz, CA), was added and incubated with the cells for 1 h at room temperature. The cells were washed twice and blocking solution (5% FBS prepared in PBS) was added and incubated with the cells for 1 h at room temperature. After another washing step, an antibody against CXCR7 (1:100), purchased from Santa Cruz Biotechnology (Santa Cruz, CA), was added and incubated with the cells for 1 h at room temperature. Images of the cells were obtained using a Carl Zeiss LSM 700 confocal microscope system (Jena, Germany).
CXCR7 expression in MSCs according to cell density

Table 1. The CXCR7 gene is highly expressed in BM-MSCs harvested at a high confluency (~90%), as determined by microarray analysis

<table>
<thead>
<tr>
<th>Abbr.*</th>
<th>Full Name</th>
<th>FC</th>
<th>Biological Function</th>
<th>Gene Ontology Category</th>
</tr>
</thead>
<tbody>
<tr>
<td>CXCR7</td>
<td>C-X-C chemokine receptor type 7</td>
<td>10.81</td>
<td>Mediated in cellular adhesion, migration, proliferation, and survival [14, 22, 24, 27]</td>
<td>GO:0070098_chemokine-mediated signaling pathway</td>
</tr>
<tr>
<td></td>
<td></td>
<td>7.31</td>
<td></td>
<td>GO:004871_signal transducer activity</td>
</tr>
</tbody>
</table>

*Abbr. = abbreviation. BM-MSCs plated at a density of 200 or 5,000 cells/cm² were incubated for 7 days, by which time they had reached 50% and 90% confluency, respectively. After harvesting, total mRNA was isolated from pooled samples of MSCs from two donors and used for microarray analysis. Microarray data were filtered by applying two criteria for significance, $P < 0.05$ and FC > 2 between culture conditions. FC refers to the comparison of MSCs harvested at a high confluency (~90%) and low confluency (~50%). Positive values indicate higher expression in MSCs harvested at a high cell density.
CXCR7 expression in MSCs according to cell density

Table 2. The number of divisions of BM-MSCs cultured under different conditions

<table>
<thead>
<tr>
<th>Condition</th>
<th>Division number</th>
</tr>
</thead>
<tbody>
<tr>
<td>200 cells/cm², Day 2</td>
<td>0.5±0.36</td>
</tr>
<tr>
<td>200 cells/cm², Day 7</td>
<td>4.69±0.42</td>
</tr>
<tr>
<td>5000 cells/cm², Day 2</td>
<td>0.37±0.2</td>
</tr>
<tr>
<td>5000 cells/cm², Day 7</td>
<td>1.57±0.36</td>
</tr>
</tbody>
</table>

Data are the mean ± SD of three separate experiments.

Figure 2. The expression level of CXCR7 in BM-MSCs harvested at a high density. A. Quantitative RT-PCR analysis of the CXCR7 gene in BM-MSCs harvested at a high density. Total RNA of BM-MSCs at passage 2, obtained 7 days after plating at a density of 200 or 5,000 cells/cm², was analyzed using qRT-PCR primers specific for CXCR7. *P < 0.01 versus cells plated at a density of 200 cells/cm² at Day 7. B. Immunoblot analysis of CXCR7 protein expression in low- and high-density MSC cultures. β-actin was used as a loading control. C. CXCR7 protein expression changes over time after seeding at a high density. β-actin was used as a loading control.

Immunohistochemistry

NOD/SCID immunodeficient mice (8-10 weeks old; Jackson Laboratories, Bar Harbor, ME) received 300 cGy of total body irradiation 24 h before intravenous injection of 2 × 10⁷ human peripheral blood mononuclear cells (hPBMCs). On the day of transplantation, MSCs were trypsinized and cultured with 1 μM CM-DiI Cell-Tracker (Invitrogen-Gibco) for 5 min at 37°C, and then for an additional 15 min at 4°C. After labeling, MSCs were washed with PBS and injected intravenously along with hPBMCs into mice. After sacrifice, the small intestines were carefully dissected using frozen sectioning techniques. Tissues were washed twice, and blocking solution (5% FBS prepared in PBS) was added and incubated with the cells for 1 h at room temperature. After another washing step, an antibody against CXCR7 (1:100) was added and incubated with the cells for 1 h at room temperature. Fluorescence images of tissues were obtained using the Carl Zeiss LSM 700 confocal microscopy system, operated with appropriate wavelengths to detect DAPI staining of nuclei (blue; Vector Laboratories, Burlingame, CA), CXCR7 (green), and CM-Dil-labeled MSCs (red). Confocal images were analyzed using LSM 700 Zen software.

Statistical analysis

Data were expressed as means ± standard deviation (SD). Statistical significance (P < 0.05) was determined using the Student's t-test.

Results

Characteristics of BM-MSCs

Human BM-MSCs were obtained from the mononuclear cell fraction of BM aspirates derived from two donors. The cells were selected based on plastic adherence to ensure the removal of any contaminating hematopoietic cells and were fibroblastic in shape (Figure 1A). Expanded cells expressed characteristic stem cell-associated surface markers (CD73, CD90, CD105, and CD166) and did not express hematopoietic antigens (CD14, CD34, and CD45) and HLA-DR (Figure 1B), indicating that expanded cells were MSCs. The expression levels of CXCR4 and CXCR7 were measured in BM-MSCs obtained from two donors. Expression of CXCR7 was high in BM-MSCs, while expression of CXCR4 was low (Figure 1C). Human myeloid leukemia HL-60 and THP-1 cells were used as a positive or negative control.

Up-regulation of the CXCR7 gene in BM-MSCs harvested at a high density

Microarray analysis was performed to investigate whether ex vivo culture conditions affected MSC characteristics. Gene expression data were compared among different cell culture conditions.
CXCR7 expression in MSCs according to cell density

Donor 1 BM-MSCs (D1 MSCs) plated at a density of 200 or 5,000 cells/cm² were incubated for 7 days, by which time they had reached 50% and 90% confluency, respectively. CXCR7 gene expression was higher in MSCs harvested at ~90% confluency than in MSCs harvested at ~50% confluency, and these findings were consistent when the microarray analysis was repeated with Donor 2 BM-MSCs (D2 MSCs) (Table 1). D1 MSCs were selected as representative cells for further study. The yields of cells plated at a density of 200 or 5,000 cells/cm² and cultured for various amounts of time are shown in Table 2. The change in the CXCR7 mRNA expression level (confirmed by qRT-PCR) was 16.2-fold (Figure 2A). The protein expression levels of CXCR7 were also investigated by western blotting. CXCR7 protein was up-regulated in MSCs harvested at ~90% confluency in comparison to MSCs harvested at ~50% confluency (Figure 2B). Additionally, expression levels of CXCR7 gradually increased with the culture duration (Figure 2C). Furthermore, confocal microscopy showed that CXCR7 expression increased as the confluency of MSCs increased (Figure 3A). Finally, the in vivo presence of BM-MSCs expressing CXCR7 was confirmed by immunohistochemistry of small intestinal tissue obtained from graft-versus-host disease (GVHD) mice injected with MSCs harvested at ~90% confluency (Figure 3B).

Validation of CXCR7 gene expression using CB-MSCs harvested at a high density

To validate the gene expression levels of CXCR7 in BM-MSCs, qPCR analysis was conducted of CB-MSCs harvested at different densities. Expression of CXCR7 was compared among CB-MSCs obtained from three donors that were plated at a density of 200 or 5,000 cells/cm² and cultured for 7 days. Expression of CXCR7 was up-regulated in the three CB-MSC donor cultures harvested at high density (~90% confluent) in comparison to cultures from the same donors harvested at a low density (~50% confluent) (Figure 4).

Discussion

Migration and engraftment of stem cells to damaged tissue are critical for successful clinical applications. The chemokine SDF-1 is a member of the chemokine C-X-C subfamily and is considered to be a critical mediator of the recruitment and migration of various stem cells including MSCs [15-24]. SDF-1 exerts its biological function by binding to its cognate receptors, CXCR4 and CXCR7 [26-28]. Thus, CXCR4 and CXCR7 expression levels in MSCs could be a critical factor for cell therapy. Our study...
CXCR7 expression in MSCs according to cell density

showed that CXCR7 expression was high but CXCR4 expression was very low, which is consistent with a previous report [29]. These results imply that CXCR7 has a more important role than CXCR4 in the migration and engraftment of naive MSCs.

MSCs are subpopulations that are isolated according to their adherence to tissue culture surfaces among cells derived from tissues [6, 8, 30]. Previous reports suggest that ex vivo expansion of MSCs is a critical factor for the results of clinical trials due to differences in their heterogeneity, growth conditions, and confluency [31, 32]. In this study, we used different plating densities and cell confluencies following culture for various amounts of time to identify the effect of ex vivo culture conditions on CXCR7 expression in human MSCs. CXCR7 expression in MSCs was affected by the culture time and cell confluency. We further explored the migration of MSCs harvested at a high confluency to damaged tissues in a GVHD animal model. CXCR7 was highly expressed in MSCs detected in the small intestine. In addition, up-regulation of CXCR7 in CB-MSCs harvested at a high density and our previous result using adipose tissue-derived MSCs [25] imply that cell culture conditions can affect CXCR7 expression in MSCs derived from different tissues. Ex vivo expansion of MSCs is an essential procedure for developing and maintaining MSCs used for cell therapies. Therefore, modulating cell culture conditions could be a favorable method for obtaining functionally qualified MSCs that can be readily adapted for more successful cell therapies. Although a low initial plating density is considered to be beneficial for ex vivo MSC expansion due to their further differentiation or expansion [33-35], MSCs harvested at a high density may be useful for the treatment of conditions associated with injured tissues, e.g., GVHD, heart infarction, and ischemia.

In conclusion, this study provides the first evidence that the cell culture density affects CXCR7 expression in MSCs, suggesting that CXCR7 expression can be modulated in MSCs by manipulating the cell density and providing useful guidelines for the collection of functionally qualified MSCs that can be more readily adapted for cell therapies.

Acknowledgements

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Disclosure of conflict of interest

None.
CXCR7 expression in MSCs according to cell density

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