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Towards In Situ Tissue Repair: Human Mesenchymal Stem Cells Express Chemokine Receptors CXCR1, CXCR2 and CCR2, and Migrate Upon Stimulation With CXCL8 but not CCL2

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Abstract The recruitment of bone marrow $CD34^-$ mesenchymal stem- and progenitor cells (MSC) and their subsequent differentiation into distinct tissues is the precondition for in situ tissue engineering. The objective of this study was to determine the entire chemokine receptor expression profile of human MSC and to investigate their chemotactic response to the selected chemokines CCL2, CXCL8 and CXCL12. Human MSC were isolated from iliac crest bone marrow aspirates and showed a homogeneous population presenting a typical MSC-related cell surface antigen profile ($CD14^-$, $CD34^-$, $CD44^+$, $CD45^-$, $CD166^+$, SH-2^+). The expression profile of all 18 chemokine receptors was determined by real-time PCR and immunohistochemistry. Both methods consistently demonstrated that MSC express CC, CXC, C and CX_3C receptors. Gene expression and immunohistochemical analysis documented that MSC express chemokine receptors CCR2, CCR8, CXCR1, CXCR2 and CXCR3. A dose-dependent chemotactic activity of CXCR4 and CXCR1/CXCR2 ligands CXCL12 and CXCL8 (interleukin-8) was demonstrated using a 96-well chemotaxis assay. In contrast, the CCR2 ligand CCL2 (monocyte chemoattractant protein-1, MCP-1) did not recruited human MSC. In conclusion, we report that the chemokine receptor expression profile of human MSC is much broader than known before. Furthermore, for the first time, we demonstrate that human MSC migrate upon stimulation with CXCL8 but not CCL2. In combination with already known data on MSC recruitment and differentiation these are promising results towards in situ regenerative medicine approaches based on guiding of MSC to sites of degenerated tissues. J. Cell. Biochem. 101: 135–146, 2007.

Key words: human mesenchymal stem cells; chemotaxis; chemokine IL-8; MCP-1; chemokine receptors; in situ tissue repair

Regenerative medicine provides novel tools for the treatment of traumatic and degenerative diseases of skeletal tissues such as cartilage [Brittberg et al., 1994; Erggelet et al., 2003]. Bone marrow mesenchymal stem cells are very prominent candidates for such cell therapy approaches since they have a high expansion capacity, avoid allogenic rejection after transplantation [Ryan et al., 2005], and show a high plasticity and therefore, have the potential to regenerate complex tissue defects [Barry and Murphy, 2004]. A few clinical studies have been reported using MSC, for example osteoarthritic joint repair [Wakitani et al., 2002].

Recent reports of the homing potential of MSC have broadened the spectrum for clinical applications of these cells. MSC are able of homing to the bone marrow and migrate and engraft into several tissues following systemic

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infusion [Koc et al., 2000; Devine et al., 2001, 2003]. Rat MSC injected intravenously or intraarterially migrate into neuronal tissue and reduce functional deficits of the brain after stroke [Chen et al., 2001], whereas intravenous delivery after myocardial infarction resulted in migration and engraftment into the ischemic myocardium [Barbash et al., 2003]. Goat MSC, delivered by intraarticular injection into the knee joint, are capable of engrafting in and repair of damaged meniscus and cartilage [Murphy et al., 2003]. Damage to the subchondral bone results in the migration of MSC from bone marrow to the injured site and in subsequent formation of a fibrocartilage-like repair tissue [Steadman et al., 2001].

However, the homing, migration and engraftment factors and mechanisms are barely characterized. Molecules like BMPs [Fiedler et al., 2002], PDGFs [Fiedler et al., 2004], IGFs [Fiedler et al., 2006], and chemokines [Luttichaux et al., 2005; Sordi et al., 2005; Honczarenko et al., 2006] are known, which show chemotactic activity on human MSC.

Chemokines are a family of small peptides that regulate proliferation, differentiation, chemotaxis, as well as other functions. The chemokine super-family is divided into four sub-families: CXC, CC, C and CX_3C chemokines, based upon the presentation of invariant cysteine (C) residues within the mature peptides. Chemokine receptors are classified as G protein-coupled receptors for CXC, CC, C or CX_3C chemokines [Murphy et al., 2000].

A few studies with contradictory results demonstrated different chemokine receptor profiles of human MSC and a chemotactic effect of particular chemokines on these cells. A dose dependent effect of CXCL12 was observed in vitro [Sordi et al., 2005; Honczarenko et al., 2006], while other groups found no effect [Luttichaux et al., 2005]. In a rat model of nerve injury, MSC transplanted into the lateral ventricles of the brain migrated to the avulsed hypoglossal nucleus, where the expression of CXCL12 and CX_3CL1 was increased, underlining a pivotal role of these molecules in homing, migration and engraftment of MSC [Ji et al., 2004].

Therefore, the aim of this study was to demonstrate the entire chemokine receptor expression profile of human MSC. Moreover, using a 96-multiwell plate chemotaxis assay, we have investigated the dose-dependent response of MSC to the CXCR1/CXCR2 ligand CXCL8 (interleukin-8), the CXCR4 ligand CXCL12 (SDF1α), and the CCR2 ligand CCL2 (MCP-1). The ulterior motive for carrying out this study was to establish a basis for in situ tissue repair based on MSC. We hypothesize that the application of chemokines will allow the recruitment of MSC from bone marrow to sites of degenerated tissues, such as bone, cartilage, myocard and brain, and their subsequent utilization for guided tissue repair. In situ regenerative medicine approaches are of special interest since it is known that the homing and migration capacity of MSC decreases during in vitro expansion [Rombouts and Ploemacher, 2003].

MATERIALS AND METHODS

Isolation and Culture of Human MSC

Human adult MSC were isolated from iliac crest bone marrow aspirates of $n = 12$ normal donors as already described [Haynesworth et al., 1992]. In brief, aspirates (3–5 ml) were washed twice with phosphate buffered saline (PBS Biochrom, Berlin, Germany) and resuspended in complete DME-medium (Biochrom) containing 10% fetal bovine serum (FBS, Lot 40F7322K, GibcoBRL, Karlsruhe, Germany). Cells were purified using a percoll gradient (Biochrom) of a density of 1.073 g/ml, were washed with PBS and then resuspended in complete DME-medium. Cells were plated at a density of 3×10^5 cells/cm^2 and cultured under standard cell culture conditions. Medium was exchanged after 72 h and every 3 days thereafter. Reaching 90% confluence, cells were detached by the addition of a solution containing 0.5% trypsin-EDTA (Biochrom) and replated at a density of 5×10^3 cells/cm^2. The study was approved by the ethical committee of the Charité-University Medicine Berlin.

Fluorescent Activated Cell Sorting (FACS) Analysis

FACS analysis was performed as described earlier [Schmitt et al., 2003]. Cells (passages 2 and 3) were washed in PBS/0.5%BSA prior incubation with titrated concentrations of primary staining reagents for 15 min on ice. Primary staining reagents unlabelled monoclonal mouse anti-human SH-2 (CD105, endoglin) was a gift from the German Rheumatism Research Center (DRFZ, Berlin, Germany),

whereas fluorescein isothiocyanate (FITC) labelled mouse anti-human CD44 and CD45, R-Phycoerythrin (PE) labelled mouse anti-human CD14 and CD166 (ALCAM) were purchased from BD-Pharmingen (Heidelberg, Germany). Monoclonal mouse anti-human CD34 labelled to PE was purchased from Miltenyi Biotech (Bergisch Gladbach, Germany). For double staining with SH-2, the following procedure was performed: cells were incubated with unlabeled SH-2, were washed and were incubated with biotinylated goat anti mouse IgG for 10 min on ice. Subsequently, cells were washed again and incubated with streptavidin coupled to cytochrome 5 (Cy5) together with FITC or PE antibodies specific for a second surface molecule, for example CD166, CD34 or CD45. Staining of other surface molecules was performed in a single step. Prior to the analysis in a LSR cytometer (Becton Dickinson, Heidelberg, Germany), cell samples were washed. Dead cells were stained with PI and then excluded. For analysis, CellQuest software (Becton Dickinson) was used.

Gene Expression Profiling of Chemokine Receptors

Total RNA from 3 donors (passage 2) was isolated using TRI Reagent LS (Sigma, Taufkirchen, Germany) following a protocol from Chomczynski [1993]. Subsequently, 5 μg total RNA were reverse transcribed using the iScript cDNA Synthesis Kit (BioRad, München, Germany) according to the manufacturer's instructions. The relative expression level of the housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used to normalize chemokine receptor expression in each sample in different concentrations. Semiquantitative real-time PCR using the i-Cycler system (BioRad) was performed with 1 μl of the cDNA sample, using the SYBR Green PCR Core Kit (Applied Biosystems, Karlsruhe, Germany). PCR conditions for all 18 chemokine receptors were hot start enzyme activation at 95°C for 10 min, 40 cycles of denaturation at 95°C for 35 s and annealing of receptor specific oligonucleotides for 45 s, and finally 1 cycle at 95°C for 30 s and at 60°C for 30 s. Oligonucleotides used for PCR are given in Table I. The specificity of oligonucleotides was verified by sequencing of resulting PCR products (Medigenomix, Inc., Martinsried, Germany). Relative quantitation of chemokine receptors was performed as described [ABI Prism 7700, 1997] and is given as a percentage of the GAPDH product.

Immunohistochemical Staining of Chemokine Receptors

For immunohistochemistry, the EnVision^{TM} + System, peroxidase (AEC) mouse kit (DAKO, Hamburg, Germany) was used. Human MSC from 3 donors (passage 3) were seeded in 8-well chamber slides (Lab-Tek, Wiesbaden, Germany), cultured for 24 h, fixed with methanol/acetone (1:1), and then were incubated for 30 min at 37°C with unconjugated primary chemokine receptor antibodies or mouse-IgG1 as control. Primary antibodies CCR1-CCR3, CCR5-7, CCR9, CXCR1-6 were purchased from R&D-System (Wiesbaden, Germany), CCR4 from BD-Pharmingen, CCR8, CCR10 and CX_3CR from DPC Biermann (Bad Nauheim, Germany). Subsequently, horseradish peroxidase (HRP)-labeled secondary antibodies were used for detection according to the manufacturer's protocol. MSC were counterstained with haematoxylin. XCR expression was not determined, since specific antibodies are not available commercially.

Chemotaxis Assay

Chemotactic responses to CCL2 (monocyte chemoattractant protein-1, MCP-1), CXCL8 (interleukin-8, IL-8) and CXCL12 (stromal derived factor-1α, SDF-1α) were measured in 96-multiwell format ChemoTx plates (Neuroprobe, Gaithersburg, more detailed info at http://www.neuroprobe.com) [Fiedler et al., 2002] with 8 μm polycarbonate membranes according to a protocol established in our group. CCL2 and CXCL8 were purchased from R&D-Systems and CXCL12 from BD-Pharmingen. Briefly, 3×10^4 human MSC in 40 μl serum free DME-medium were seeded in triplicates in the upper wells each. The lower wells were supplied with 1–10^3 nM recombinant CCL2, CXCL8 or CXCL12 in 35 μl serum free DME-medium and the chambers were incubated for 20 h at 37°C. After removal of non-responding human MSC on top of the filter, cells that migrated through the membrane were fixed in ethanol/acetone, stained with Hemacolor (Merck, Darmstadt, Germany) and enumerated microscopically by counting the number of stained cells in three representative fields. Medium without chemokines (negative) and medium containing fetal calf serum (positive) served as controls. To test

TABLE I. Oligonucleotides Used for Chemokine Receptor Gene Expression Analysis

Subfamily	Gene	EMBL-database	Oligonucleotide (5′->3′)	Product size (b.p.)
CC	CCR1	NM001295	For ACCATAGGAGGCCAACCCAAAATA	103
			Rev TCCATGCTGTGCCAAGAGTCA	
	CCR2	NM000647	For CTACCTTCCAGTTCCTCATTTTT	100
			Rev ACATTTACAAGTTGCAGTTTTCAGC	
	CCR3	NM178329	For TTTGTCATCATGGCGGTGTTTTTC	169
			Rev GGTTCATGCAGCAGTGGGAGTAG	
	CCR4	NM005508	For GAGAAGAAGAACAAGGCGGTGAAGA	200
			Rev GGATTAAGGCAGCAGTGAACAAAAG	
	CCR5	NM000579	For CAACCACAGGCAGCATTTAGCAC	147
			Rev GGCAGGCAGCATCTTAGTTTTTCAG	
	CCR6	NM031409	For CTGCCTGAACCCTGTGCTCTACG	171
			Rev TTATCTGCGGTCTCACTGGTCTGC	
	CCR7	NM001838	For GCCGAGACCACCACCACCTT	105
			Rev AGTCATTGCATCTGCTCCCTATCC	
	CCR8	NM005201	For AAGCCCCTGTGATGCGGAACT	123
			Rev CAGACCACAAGGACCAGGATGAC	
	CCR9	NM031200	For TATACAGCCAAATCAAGGAGGAATC	137
			Rev CATGACCACGAAGGGAAGGAAG	
	CCR10	NM016602	For GGGCTGGAGTCTGGGAAGTGC	183
			Rev ACGATGACGGAGACCAAGTGTGC	
CXC	CXCR1	NM000634	For CTGAGCCCCAAGTGGAACGAGACA	152
			Rev GCACGGAACAGAAGCTTTATTAGGA	
	CXCR2	NM001557	For CAATGAATGAATGAATGGCTAAG	118
			Rev AAAGTTTTCAAGGTTCGTCCGTGTT	
	CXCR3	NM001504	For CCCGCAACTGGTGCCGAGAAAG	148
			Rev AGGCGCAAGAGCAGCATCCACAT	
	CXCR4	NM003467	For ATCCCTGCCCTCCTGCTGACTATTC	231
			Rev GAGGGCCTTGCGCTTCTGGTG	
	CXCR5	NM032966	For TCCCCTCCTCACTCCCTTCCCATAA	224
			Rev CCTGCGGTTCCATCTGAGTGACATC	
	CXCR6	NM006564	For TTGTTTATAGCTTGCGCATTCTCAT	189
			Rev ATCCCCCTTGGTTTCAGCATTCTT	
CX3C	CX3CR	NM001337	For ATAGATTCCCCATTGCCTCCTC	120
			Rev GGTTTTTCTATTTCCCTTACTGG	
C	XCR	NM005283	For CATCATGACCATCCACCGCTACC	129
			Rev TCGAGGATGGAGGACAGGATGC	

The table shows the sequences of oligonucleotides used to demonstrate the expression of all 18 CC, CXC, CX_3C and C chemokine receptors, the EMBL-database accession number of the sequence used for oligonucleotide design, and the real-time RT-PCR product size in base pairs (b.p.).

for CXCL8 and CXCL12-induced chemokinesis, in an additional experiment these chemokines were added in same concentrations to both the upper and lower wells in triplicates [Zigmond and Hirsch, 1973].

RESULTS

Isolation, Culture and Flow Cytometric Analysis of Human MSC

Human bone marrow-derived cells consisted mostly of erythrocytes and nonadherent growing haematopoietic cells. About day 3, cells adhered and nonadherent cells were removed due to the exchange of culture medium. During primary cell culture, attached MSC stretched and took shape of typical fibroblast-like cells. Morphologically, expanded MSC (P2, passage 2) presented a stable fibroblast-like phenotype (Fig. 1A). MSC cultures (P2) were assayed routinely for the presence of MSC-related cell surface antigens by flow cytometric analysis, and displayed a homogenous population. They were uniformly positive for SH-2 (CD105, endoglin), for the activated leukocyte cell adhesion molecule ALCAM (CD166) (Fig. 1B, 97% SH-2/CD166 double positive), and the hyaluronan receptor CD44 (data not shown). MSC were negative for haematopoietic antigens such as the lipopolysaccharide receptor CD14 (data not shown) and the haematopoietic stem cell marker CD34 (Fig. 1C). Most cultures also stained negative for the leukocyte common antigen CD45. However, some cultures were slightly double positive for CD45/SH-2 (Fig. 1D, 84% $CD45^-/SH\text{-}2^+$).

Expression of Chemokine Receptors by Human MSC

In this study, the entire chemokine receptor expression profile of human MSC derived from six independent donors was determined on the level of gene expression (P2, $n = 3$) and immunohistochemistry (P3, $n = 3$). Semi-quantitative real-time PCR and subsequent sequence analysis of the PCR products demonstrated that

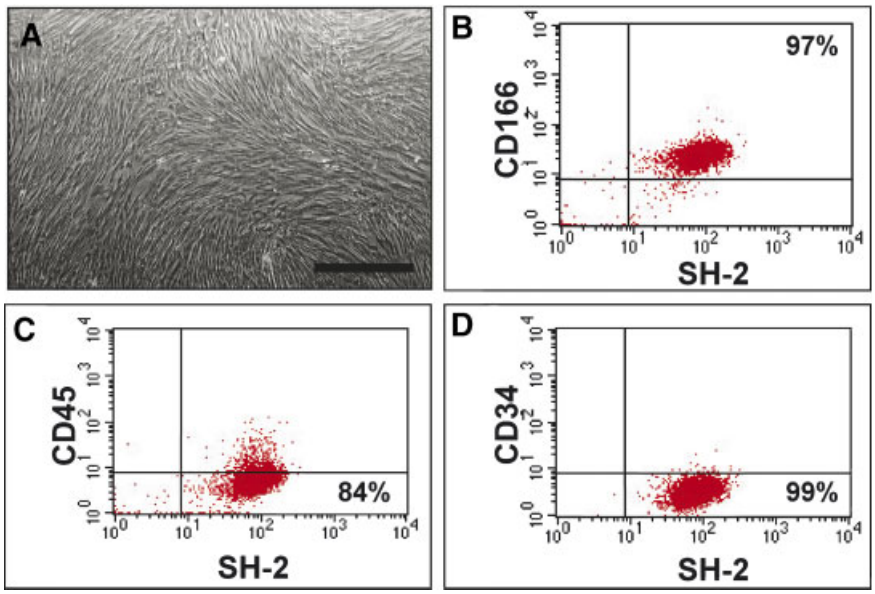

Fig. 1. Morphology and FACS analysis of human MSC. Human mesenchymal stem- and progenitor cells (MSC) were isolated from bone marrow aspirates using percoll density centrifugation and expanded in selected culture medium. **A**: During cell culture MSC adhered to the culture plate, stretched and presented at least up to passage 2 a stable fibroblast-like phenotype. **B**–**D**: Flow cytometry demonstrates that MSC are positive for reactivity to MSC-related antigens SH-2 and ALCAM (activated leukocyte cell adhesion molecule). They are negative for haematopoietic cell markers like CD34 and CD45 (84% CD45-/SH-2+). Scale bar corresponds to 200 μm. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

human MSC express a much broader panel of receptors for chemokines of all four subfamilies than reported so far (Fig. 2). The relative expression level of the particular receptors was calculated as a percentage of the expression of the housekeeping gene GAPDH.

In general, the expression of all chemokine receptors was very low and between 10^{-4}% and

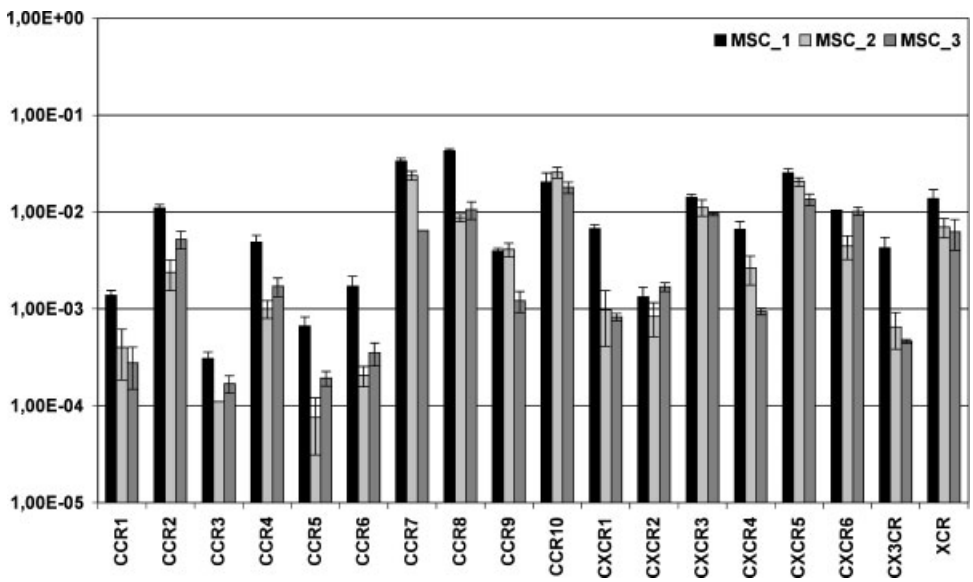

Fig. 2. Semi-quantitative chemokine receptor gene expression of human MSC. More than 50 human chemokines mediate their effects through one or more of 18 chemokine receptors. These receptors are classified as receptors for CC, CXC, C and CX_3C chemokines. Real-time RT-PCR expression analysis of three independent human MSC donors demonstrates a wide spectrum of chemokine receptors expressed by these cells. On mRNA level human MSC express the chemokine receptors CCR2, CCR8, CXCR1-CXCR3 and XCR, receptors whose expression has not been reported so far. In general, the expression of all 18 receptors was very low. The relative expression level of the particular receptors was calculated as a percentage of the expression of the housekeeping gene GAPDH.

10^{-1}% of GAPDH. We detected the expression of receptors such as CCR1, CCR4, CCR6, CCR7, CCR9, CCR10, CXCR4, CXCR5, CXCR6 and CX_3CR (Fig. 2), whose expression has already been reported for human MSC. Moreover, MSC expressed CCR2, CCR8, XCR, CXCR1, CXCR2 and CXCR3 (Fig. 2), chemokine receptors whose expression has not been reported so far. CCR2, CCR7, CCR8, CCR10, CXCR3, CXCR5 and XCR represented the strongest expressed receptors (about 10^{-2}% of GAPDH). Generally, the profile showed a variable expression of most of the receptors.

Immunohistochemical staining of MSC demonstrated, that these cells also present chemokine receptors for CC, CXC and CX_3C chemokines on the protein level (Fig. 3). All three MSC donors stained positive for all six known CXCR receptors confirming the qualitative expression of CXCR1, CXCR2 and CXCR3 (Fig. 3) (Table II). CCR2 was expressed in abundance as shown by gene expression analysis, and was detected in two out of three donor immunohistochemically. CCR8 and CCR9 showed an expression independent of the donor, and CCR4 was not detected. The expression of most CC receptors varied between donors (Fig. 3) (Table II).

Dose-Dependent Chemotactic Effect of CCL2, CXCL8 and CXCL12

In our in vitro recruitment screen, a 96-well plate chemotaxis assay using bone marrow-derived human MSC (passages 2 and 3) was performed to assess the functional activity of the chemokines CCL2, that binds to the CCR2 receptor, of CXCL8, a ligand for CXCR1 and CXCR2, and of CXCL12, the ligand for the chemokine receptor CXCR4. In general, the number of cells migrated in the positive controls (FBS instead of chemokine) was reproducible between 4×10^3 and 5×10^3. As shown in Figure 4, although human MSC express the CCR2 receptor in vitro its ligand CCL2 caused no chemotactic effect on these cells (Fig. 4AI–III). The number of migrating cells was lower than in the negative controls. The average numbers of cells that migrated in the negative controls are given by the two lines named UL (upper limit, average number of migrated cells at 0 nM chemokine concentration plus standard deviation) and lower limit (LL, average number of migrated cells at 0 nM chemokine concentration minus standard deviation). In contrast, CXCL8 showed a dose-dependent chemotactic effect on human MSC (Fig. 4BI–III). The dose-effect maximum was measured at 750 nM for three of three MSC donors. Hundred nanomolar was the minimum concentration of CXCL8 that chemoattracts MSC. A dose-dependent activity of CXCL12 on MSC and the expression of its receptor CXCR4 have already shown before, but with contradictory results. We verified the expression of CXCR4 on the mRNA level (Fig. 2) and protein level (Table II), and in addition showed that CXCL12 caused migration of human MSC. Analysing three donors, the highest response was measured using this chemokine in a concentration of 750 nM (one donor) (Fig. 4CI) or between 250 and 1000 nM (two donors) (Fig. 4CII–III). Similar to CXCL8, 100 nM was the minimum concentration of CXCL12 that chemoattracts human MSC.

To show that the migratory response was due to site-directed chemotaxis and not to chemokinesis, in one migratory assay CXCL8 and CXCL12 were added to both the upper and lower wells, and therefore the concentration gradients were eliminated. For 750 nM CXCL8 in the lower well, we found 1437 ± 11.3 migrated MSC. After elimination of the gradient, no migrated cells were measured demonstrating chemotaxis but no chemokinesis. Using 500 nM CXCL12 in the lower well, we counted 420 ± 80.7 migrated cells and in the chemokinesis assay we measured 8 ± 11.3 migrated cells, again indicating chemotaxis but no chimokinesis. The number of cells migrated in the negative control was 21.4 ± 24.5.

DISCUSSION

Regenerative medicine approaches have progressed from the 'bench to bedside'. Beyond implantation of cell suspensions [Brittberg et al., 1994], advanced Tissue engineering grafts comprising cultured cells are clinically applied. Due to a limited proliferation capacity of many adult organ specific cells and a loss of functional quality during expansion in vitro, much attention has been drawn to stem cells such as MSC [Caplan and Bruder, 2001]. In most of the few MSC based clinical approaches, these cells were delivered as a cell suspension for cardiac or meniscal tissue repair (Osiris Therapeutics Inc., Baltimore) or for co-transplantation with haematopoietic stem cells in, for example haematologic malignancy patients

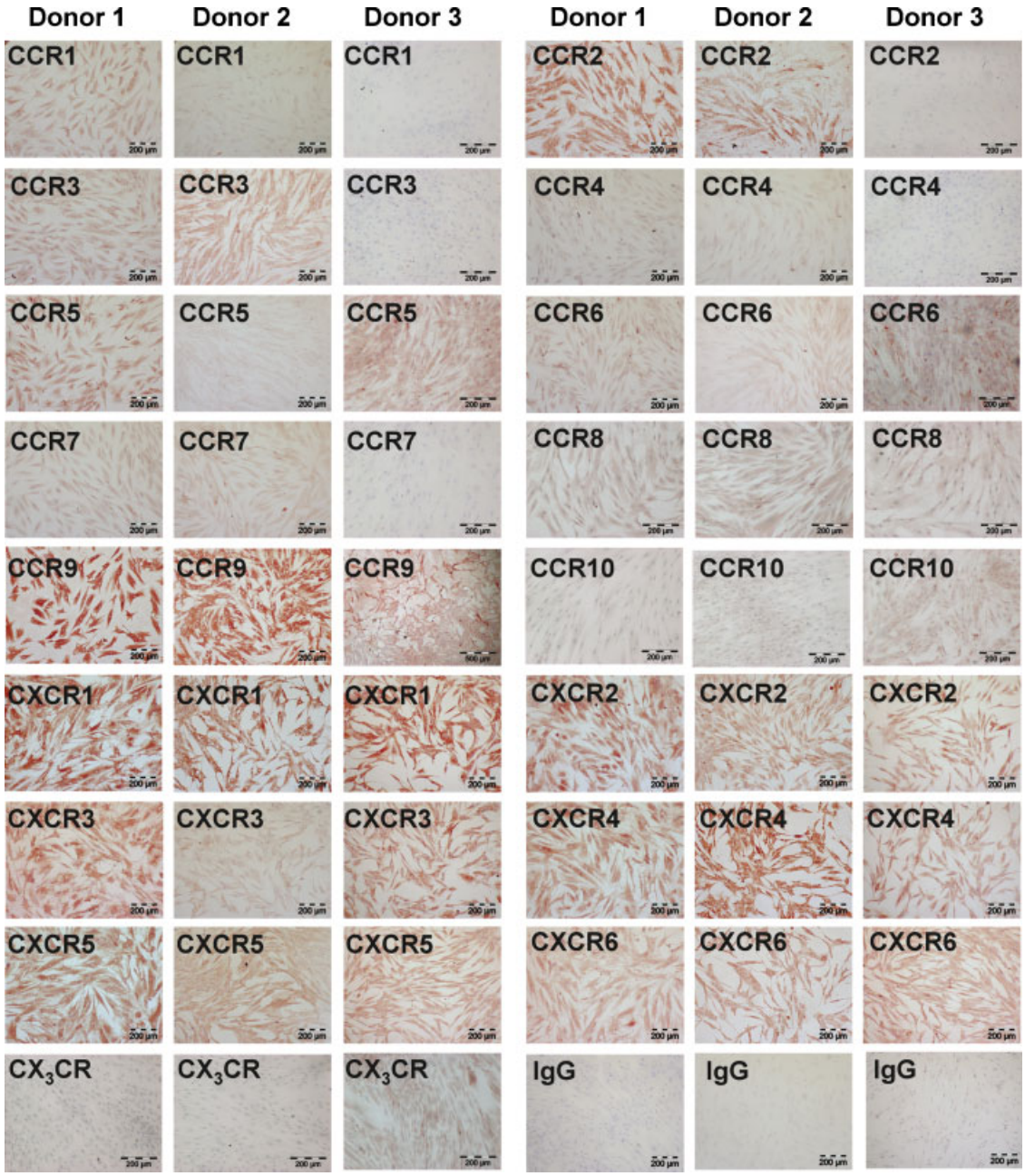

Fig. 3. Immunohistochemical staining of the chemokine receptors CCR1-10, CXCR1-CXCR6 and CX_3CR. Human mesenchymal stem- and progenitor cells (MSC) present distinct chemokine receptors for CC, CXC and CX_3C chemokines also on the protein level (n = 3 donor) Interestingly, they stained positive for CCR2, CCR8, CXCR1, CXCR2 and CXCR3, receptors whose expression was unknown so far. IgG, negative control. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

[Lazarus et al., 2005]. Such therapies are promising because of the homing, migration and engraftment potential of MSC [Koc et al., 2000].

With respect to our rationale to establish the basis for in situ tissue repair based on biomaterials, chemokines and human bone marrow $CD34^-$ MSC, here we reported their entire chemokine receptor expression profile on the mRNA and protein level. Recently, the expression of CCR1, CCR7, CCR9, CXCR4-CXCR6 [Honczarenko et al., 2006], CCR1, CCR4, CCR7, CCR9-CCR10, CXCR5 [Luttichaux et al., 2005], and CCR1, CCR7, CXCR4, CXCR6 and CX_3CR [Sordi et al., 2005] has been reported. We found that MSC also express CCR2, CCR8, CXCR1,

TABLE II. Immunohistochemical Chemokine Receptor Profile of Human MSC

Chemokine receptor	Donor 1	Donor 2	Donor 3
CCR1	+	−	−
CCR2	+	+	−
CCR3	+	+	−
CCR4	−	−	−
CCR5	+	−	+
CCR6	+	−	+
CCR7	+	+	−
CCR8	+	+	+
CCR9	+	+	+
CCR10	−	−	+
CXCR1	+	+	+
CXCR2	+	+	+
CXCR3	+	+	+
CXCR4	+	+	+
CXCR5	+	+	+
CXCR6	+	+	+
CX_3CR	−	−	+
XCR	n.d.	n.d.	n.d.

CCR1-CCR10, CXCR1-CXCR6 and CX_3CR antibodies were used to study the chemokine receptor expression profile on protein level. The table shows the expression profiles of three independent MSC donors (passage 3). For XCR no commercial antibody was available. +, positive antibody staining; −, no antibody staining; n.d., not determined.

CXCR2, CXCR3 and XCR. Thus, they express a broad spectrum of receptors of all four chemokine sub-families CC, CXC, C and CX_3C. Interestingly, in case of acute diseases, tissues like myocard or brain up-regulates their secretion of distinct chemokines such as CXCL12 and CX_3CL1 [Wang et al., 2002; Ji et al., 2004; Vandervelde et al., 2005]. Moreover, human cartilage chondrocytes secrete several chemokines that might be potent to attract human MSC in vivo [De Ceuninck et al., 2004]. Antibody microarrays demonstrated the secretion of more than 20 chemokines such as the CCR2 ligand CCL2, the CCR8 ligand CCL1, ligands for CXCR1 (CXCL5, CXCL8) and CXCR2 (CXCL1, CXCL5, CXCL8), and the CXCR3 ligand CXCL10 by articular chondrocytes. However, divergent chemokine receptor profiles reported by different groups may be due to variations between donors (as expected for primary cells), the state of the particular donor, and the isolation and expansion protocol. In addition, due to the lack of an MSC specific antibody, exact determination of the MSC culture homogeneity in general is not possible. Therefore, varying culture homogeneity may influence results obtained in MSC gene- and protein expression, and migration assays. In this study, MSC were isolated using percoll centrifugation [Pittenger et al., 1999] and expanded in selected medium [Lennon et al., 1996]. These cells showed the typical morphological appearance known from bone marrow-derived MSC, and a characteristic expression of MSC-related cell surface antigens SH-2 (endoglin, CD105) [Barry et al., 1999], ALCAM (SB-10, CD166) [Bruder et al., 1998], CD14, CD34 and CD45. Other antibodies such as SH-3 (CD73) [Barry et al., 2001], Stro-1 [Simmons and Torok-Storb, 1991] and CD90 (Thy-1) also react with MSC. All mentioned antibodies react with a variety of other cell types and this remains an obstacle in studying migration, homing and engraftment mechanisms of endogenous and implanted MSC. For in situ tissue repair using chemoattractants in combination with differentiation factors, little is known about the role of chemokines and their receptors during differentiation and embryonic development. It has been reported that CXCL1 is expressed in different tissues, including the condensing mesenchyme that gives rise to bone and cartilage in murine embryonic development [Luan et al., 2001]. The pattern of CXCL1 expression follows that of CX2CR, indicating that CXCL1/CX2CR interactions are important for the development of skeletal tissues. However, the normal development of the skeletal elements in CXCR2 double knockout mice suggests that the function of CXCR2 in skeletal development must be redundant. It is known that multiple chemokine receptors often address similar effector signalling pathways, which makes it difficult to determine the distinct role of a particular receptor in differentiation and migration processes [Rollins, 1997].

Recently, the homing abilities of uncultured and culture expanded MSC were compared in a mouse model [Rombouts and Ploemacher, 2003]. Twenty-four hours after injection into the lateral tail vain, about 60% of primary MSC were detectable in the bone marrow, whereas 48 h culture-expanded MSC could not be detected anymore. Honczarenko et al. [2006] expanded human MSC for more than 10 passages and found that the decrease of the homing potential is in concordance with a loss of surface expression of chemokine receptors and a lack of chemotactic response to chemokines. This implies the importance of in situ tissue repair approaches, in which no cells have to be isolated and grown in vitro.

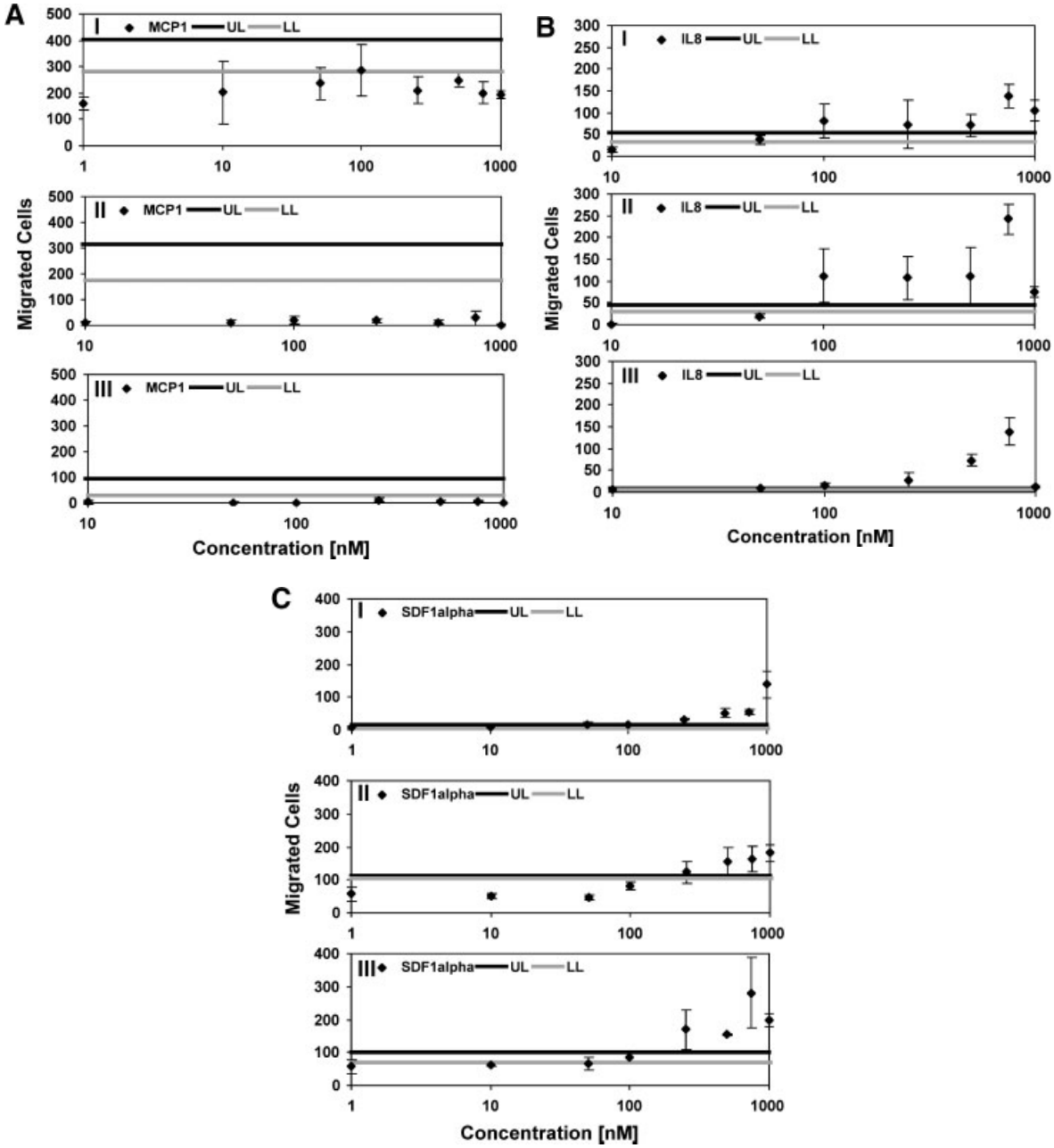

Fig. 4. Chemotactic response of human MSC to CCL2, CXCL8 and CXCL12. Ninety six-multiwell plate chemotaxis assays were performed to assess the dose-dependent effect of CCL2 (MCP-1), CXCL8 (interleukin-8) and CXCL12 (SDF-1α) on human MSC. **A**(**I**–**III**): CCR2 caused no chemotactic response on MSC. **B**(**I**–**III**): In contrast, for the first time, a migratory effect of CXCL8 on human MSC is shown here. Optimal concentration was 750 and 100 nM was the minimum concentration of CXCL8 that chemoattracts human MSC. **C**(**I**–**III**): In addition, CXCL12 showed a dose-dependent activity on these cells. Concentrations of about 250–1000 nM resulted in the highest response. (**UL**: upper limit, average number of migrated cells at 0 nM chemokine concentration + SD, LL: lower limit, average number of migrated cells at 0 nM chemokine concentration—SD, SD: standard deviation).

As shown here, CXCL8 and CXCL12 caused a dose-dependent response on human MSC. Moreover, we demonstrated that the migratory response was directional and clearly dependent on a CXCL8 and CXCL12 gradient confirming that MSC movement was due to chemotaxis and not to chemokinesis. This has already been shown for CXCL12 and some other chemokines [Honczarenko et al., 2006]. In general, the number of migrated cells was low and donor

and assay dependent. This was in concordance with assays published before not only for MSC [Wang et al., 2002].

The importance of CXCL12 and its receptor CXCR4 is obvious. Knockout studies in null mice on either CXCL12 or CXCR4 resulted in the same phenotype, they die perinatally showing severe bone marrow failure [Zou et al., 1998]. It is assumed that the neonatal bone marrow failure is a consequence of impaired migration of stem cells, more precisely, CXCL12 might be important for the movement of progenitor cells from fetal liver to bone marrow during embryogenesis. The injection of CXCL12 in the rat cerebral cortex area significantly induces the accumulation of MSC at this area in vivo [Ji et al., 2004]. A very recent paper stated that MSC express CXCR1 and CXCR2 on mRNA level [Lisignoli et al., 2006]. Although both receptors are mainly known from acute inflammation and innate immunity [Murphy et al., 2000], we have demonstrated their expression by human MSC using real-time PCR, immunohistochemistry and a functional assay. As shown here, the inflammatory chemokine CXCL8 (IL-8), which is known as a potent chemoattractant for neutrophils [Spanaus et al., 1997], also caused a dose-dependent chemotactic response to MSC. Interestingly, MSC as well as other cells like chondrocytes continuously secrete CXCL8 in vitro. A possible migratory effect of this chemokine on MSC in vivo has to be investigated in future studies. In chemotaxis assays using other cell types, CXCR2 was relatively non-selective for CXCL8 when compared to other ligands such as CXCL1 and CXCL5, whereas CXCR1 was highly selective for CXCL8 [Lee et al., 1992; Loetscher et al., 1994].

Activated T-cells, monocytes and basophils represent important targets for CCL2 (MCP-1) [Lahrtz et al., 1998]. Although expression of its receptor CCR2 was shown here, CCL2 caused no dose-dependent chemotactic response on human MSC. In rats, CCL2, not present in normal brain, is rapidly up-regulated following middle cerebral artery occlusion, and promotes the migration of injected bone marrow mesenchymal cells to the sites of injury [Wang et al., 2002]. The in vivo role of CCL2 and other chemokines, chemoattracting human MSC in vitro such as CCL3, CCL5, CCL17, CCL19, CCL21, CCL25, CCL28, CXCL10, CXCL12, CXCL13, CXCL16, CXCL13 and CX_3CL1 [Luttichaux et al., 2005; Sordi et al., 2005; Honczarenko et al., 2006], have to be investigated in more detail in further studies. For example, the observed optimal concentrations for CXCL8 and CXCL12 were high and not physiological. Therefore, appropriate concentrations should be further investigated in vivo.

In conclusion, we demonstrated that the chemokine receptor expression profile of normal donor-derived human MSC is much broader than presented before. Moreover, we showed that MSC dose-dependently respond to CXCL12 (SDF-1α), and, for the first time, also to CXCL8 (interleukin-8). In contrast, they do not migrate following stimulation with CCL2 (MCP-1). Although these promising results and the already reported data represent the initial steps on the way towards new in situ tissue therapies based on chemokine induced MSC migration into distinct defect sites, clearly, the chemotactic activity of other chemokines and combinations thereof have to be analysed in vivo. Furthermore, since distinct chemokines and their receptors take part in, for example inflammatory processes, MSC-chemokine interactions need to be further investigated.

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