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Chemokine profile of synovial fluid from normal, osteoarthritis and rheumatoid arthritis patients: CCL25, CXCL10 and XCL1 recruit human subchondral mesenchymal progenitor cells

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SUMMARY

Objective: The microfracture technique activates mesenchymal progenitors that enter the cartilage defect and form cartilage repair tissue. Synovial fluid (SF) has been shown to stimulate the migration of subchondral progenitors. The aim of our study was to determine the chemokine profile of SF from normal, rheumatoid arthritis (RA) and osteoarthritis (OA) donors and evaluate the chemotactic effect of selected chemokines on human subchondral progenitor cells.
Method: Chemokine levels of SF were analyzed using human chemokine antibody membrane arrays. The chemotactic potential of selected chemokines on human mesenchymal progenitors derived from subchondral cortico-spongious bone was tested using 96-well chemotaxis assays. Chemokine receptor expression of subchondral progenitors was assessed by real-time gene expression analysis and immunohistochemistry.
Results: Chemokine antibody array analysis showed that SF contains a broad range of chemokines. Ten chemokines that showed significantly reduced levels in RA or OA compared to normal SF or robustly high levels in all SF tested were used for further chemotactic analysis. Chemotaxis assays showed that the chemokines MDC/CCL22, CTACK/CCL27, ENA78/CXCL5 and SDF1α/CXCL12 significantly inhibited migration of progenitors, while TECK/CCL25, IP10/CXCL10 and Lymphotactin/XCL1 effectively stimulated cell migration. MCP1/CCL2, Eotaxin2/CCL24 and NAP2/CXCL7 showed no chemotactic effect on subchondral progenitors. Gene expression and immuno-histochemical analysis of corresponding chemokine receptors document presence of low levels of chemokine receptors in subchondral progenitors, with the CXCL10 receptor CXCR3 showing the highest expression level.
Conclusion: These results suggest that SF contains chemokines that may contribute to the recruitment of human mesenchymal progenitors from the subchondral bone in microfracture.

Introduction

Bone marrow stimulating techniques like drilling or microfracture are frequently used first-line treatment options for cartilage lesions. These techniques provide access to the subchondral bone marrow and allow mesenchymal progenitor cells from the marrow to migrate into and populate the defect. Subsequently, progenitors form a non-hyaline cartilaginous repair tissue[1–6]. The cellular mechanisms that underlie recruitment of progenitors to the defective site and guiding of the progenitors towards repair tissue formation are scarcely understood. Mesenchymal progenitors from the subchondral bone marrow may enter the defect passively by the blood flow and form cartilaginous repair tissue upon stimulation with angiogenic factors, growth and differentiation factors as well as cytokines that are released from the subchondral bone[4,7]. Recently, it has been shown that synovial fluid (SF) may also induce cartilage tissue formation by mesenchymal progenitors as shown by chondrogenic differentiation

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of equine stem cells from tibial bone marrow or of rabbit perichondral fibrocytes treated with SF[8,9]. In a previous study, we have shown that SF recruit subchondral mesenchymal progenitor cells and therefore may contribute to the population of the defective site by progenitors in microfracture. These multi-potent progenitor cells derived from the subchondral spongious bone, called cortico-spongious progenitors (CSP), have been shown to migrate upon stimulation with human SF, with normal and osteoarthritis (OA) SF showing an elevated recruiting potential compared to SF obtained from rheumatoid arthritis (RA) donors[10]. Obviously, SF may play an important role in progenitor cell recruitment and differentiation in microfracture and, in addition, provides the articular environment with hyaluronan, growth factors, inflammatory cytokines as well as chemokines, depending on the inflammatory, normal or degenerative condition of the joint[11–14].

Chemokines are chemotactic cytokines and comprise a family of more than 50 members that are structurally related to one of the four subgroups of C, CC, CXC, and the CX_3C chemokines and signal through binding of G protein coupled seven transmembrane-spanning serpentine receptors[15,16]. In addition to their immunological function in inflammation and homing, the pattern of chemokines and chemokine receptors in normal and OA chondrocytes as well as in mesenchymal stem cells (MSC) differentiating towards the chondrocyte phenotype suggests that chemokines have an impact in cartilage homeostasis and release of matrix-degrading enzymes in normal cartilage remodeling as well as in cartilage destruction[17,18,19]. In recent years, human MSC have been shown to express a broad range of chemokine receptors and a variety of chemokines have been reported to stimulate the migration of MSC obtained from bone marrow aspirates[20–23]. However, the role of chemokines or chemo-attractants in mesenchymal progenitor migration from the subchondral bone marrow to sites of cartilage defects in microfracture is unclear.

In this study, we analyzed the chemokine profile of SF derived from normal, OA and RA donors that have been shown previously to recruit human subchondral progenitor cells[10]. High level chemokines and chemokines with reduced levels in RA and OA SF compared to normal SF were tested for the recruitment of human mesenchymal progenitor cells derived from the subchondral spongious bone. Progenitors showed a distinct set of chemokine receptors and were recruited by the chemokines CCL25, CXCL10 and XCL1, while the chemokines CCL22, CCL27, CXCL5 and CXCL12 had an inhibitory and CCL2, CCL24 and CXCL7 had no effect on progenitor cell migration. Therefore, it is suggested that chemokines of the SF may be involved in the migration of multi-potent progenitors from the subchondral bone marrow to the defective site in microfracture.

Method

Isolation and cultivation of human cortico-spongious progenitor cells

Human subchondral progenitor cells CSP were isolated from subchondral cortico-spongious bone containing marrow (one female, three males, age 40–68 years) from the lateral tibia head during high tibial closed wedge osteotomy as described previously. CSP have a multi-potential differentiation capacity and are routinely tested for typical stem cell-like cell-surface antigen pattern with presence of SH2, CD73, CD90, and CD166 as well as absence of CD3, CD14, CD34, and CD45[24].

In brief, spongious bone was cut into small fragments, digested for 4 h at 37°C using 256 U/ml collagenase XI (Sigma) and placed in cell culture flasks (Becton Dickinson) with DME-medium (Biochrom) containing 10% human serum (German Red Cross), 100 U/ml penicillin, 100 μg/ml streptomycin, 2 mM L-glutamine (all Biochrom) and 2 ng/ml fibroblast growth factor-2 (Tebu-bio). At 80–90% confluence, CSP were harvested using trypsin–EDTA in PBS (0.05% v/v, Biochrom) and re-plated at a density of 6,000 cells/cm^2. Medium was exchanged every 2–3 days. The study was approved by the ethics committee of the Charité-Universitätsmedizin Berlin.

Collection of SF

SF used in the current study has already been shown to recruit human CSP in a previous study[10]. In brief, RA SF was obtained by joint puncture (five females; mean age 45.4 years; mean DAS 28 6.6; mean ESR (1 h) 74.2; mean CrP 6.96 mg/dl) from patients diagnosed according to the revised American College of Rheumatology criteria for the Classification of RA[25]. One donor with RA received disease modifying anti-rheumatic drugs (DMARD). Two donors had DMARD and steroidal anti-inflammatory drugs (SAID), one donor received SAID and non-steroidal anti-inflammatory drugs (NSAID), and one donor had only NSAID. None of the patients received intra-articular therapy. OA SF was obtained by joint puncture (five females; mean age 62.0 years; mean ESR (1 h) 12; mean CrP 0.7 mg/dl) from patients diagnosed according to the American College of Rheumatology criteria for the classification and reporting of OA[26]. Three of the donors with OA received NSAID and two donors had no medication at the time of joint puncture. ND SF was obtained post mortem from organ donors (three females, two males; mean age 53.2 years) without joint diseases. All procedures were performed in consent with the ethics committee of the Charité-Universitätsmedizin Berlin.

Human chemokine antibody membrane array analysis

The levels of 38 chemokines were analyzed in individual samples of SF derived from ND ($n = 5$), OA ($n = 5$) and RA ($n = 5$) using human chemokine antibody membrane arrays (RayBiotech) according to the manufacturer's recommendations ($n = 15$ arrays in total). The configuration of the chemokine antibody membrane array is given in Fig. 1(D). For normalization, the protein content of SF was measured using the BCA (bicinchoninic acid) assay (Sigma). After blocking, membranes were incubated for 2 h with 0.6 mg/ml SF at 20°C. Detection of chemokines was performed according to the manufacturer's recommendations by biotin-conjugated antibodies raised against the particular chemokines and horseradish peroxidase-conjugated streptavidin. For detection by chemiluminescence, the membranes were briefly exposed to X-ray films (Amersham) for 1.0 min. Images were digitized at a resolution of 300 d.p.i. and spot intensity was determined by densitometric measurement. In brief, a color was defined representing the background color of the array in a given area (negative control). For each spot, the number of stained and unstained pixels was determined within a given area. For normalization of arrays, signal values of chemokines were divided by the mean value of the positive control and multiplied with 100. A value of 100 represents a spot intensity as shown in the positive control, while a value of zero represents the spot intensity as seen in the negative control.

Analysis of chemokine mediated chemotaxis

Chemotactic activity of the chemokines (all PeproTech) CXCL5 (ENA78), CXCL7 (NAP2), CXCL10 (IP10), CXCL12 (SDF1α), CCL2 (MCP1), CCL22 (MDC), CCL24 (Eotaxin2), CCL25 (TECK), CCL27 (CTACK), and XCL1 (Lymphotactin) was measured in 96-well chemotaxis assays with 8 μm polycarbonate membranes (Neuroprobe), in triplicates using three independent CSP preparations each ($n = 9$ assays per chemokine and dose). Briefly, 3×10^4 CSP (passage three) were re-suspended in DME-medium containing 0.1% FBS and seeded in the upper wells. In the lower wells, 0 nM–1,000 nM of

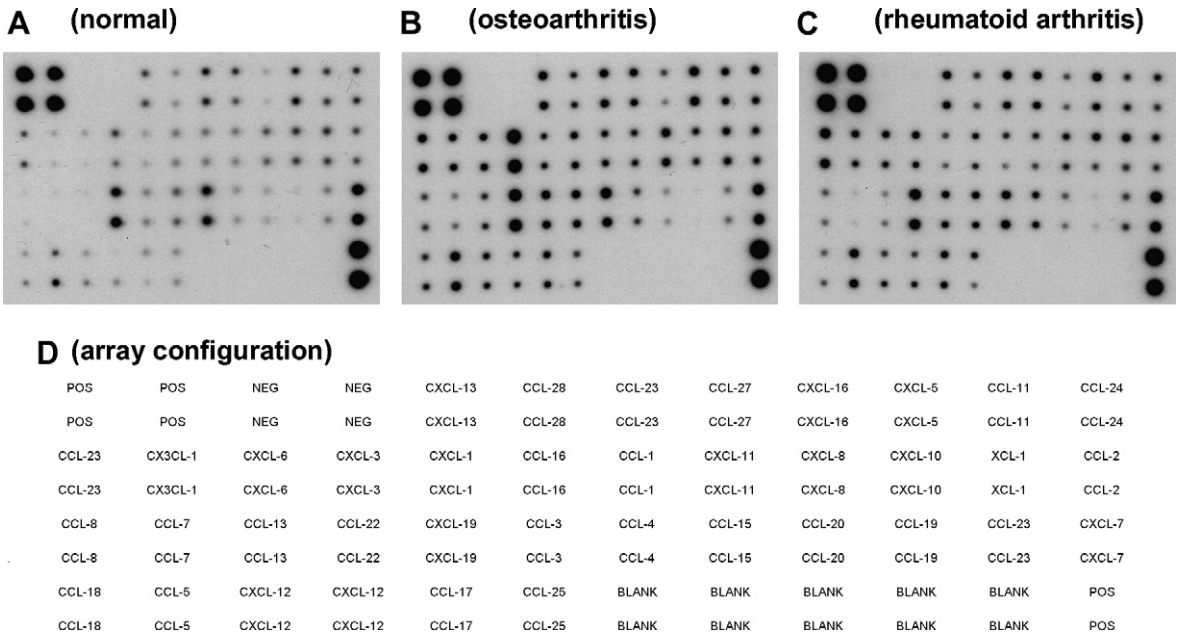

Fig. 1. Chemokine profile of human SF. Representative chemokine antibody array membranes showing the chemokine profile of SF derived from normal (**A**), OA (**B**) and RA (**C**) donors. The respective chemokines were measured in duplicates per array (**D**). In total, per group $n = 5$ SF were analyzed. Note that levels of a given chemokine can be compared from array to array, but not the levels of one chemokine to the other. Positive controls on the array membrane are streptavidin conjugated horseradish peroxidase and negative controls are bovine serum albumins.

the respective chemokine in DME-medium containing 0.1% FBS was given. Chemokinesis was excluded by testing cell migration with 50 nM or 1,000 nM of the chemokine in DME-medium containing 0.1% FBS, added to the upper and lower wells. The chambers were incubated for 20 h at 37°C. After removal of non-responding cells on top of the filter, CSP that migrated through the membrane were fixed in methanol/acetone, stained with Hemacolor (Merck) and enumerated microscopically by counting the number of stained cells in three representative fields. Data were normalized by calculating the 'migration index' (ratio between migrated CSP upon chemokine treatment and migrated CSP in the absence of the chemokine) and statistical analysis was performed.

Immuno-histochemistry

Staining of chemokine receptors was performed in duplicates. CSP (passage three) were seeded in chamber-slides at a density of 5,000 cells/cm^2 and cultured for 24 h in the presence of 10% human serum from whole blood. Cells were fixed with ice-cold methanol/acetone (1:1 v/v) and incubated with the primary mouse anti-human CXCR3 (RD Systems), mouse anti-human CCR9 (RD Systems), or rabbit anti-human XCR1 (Acris Antibodies) for 40 min. Rabbit or mouse IgG (DAKO) served as controls. Subsequently, cells were processed using the EnVision System Peroxidase Kit (DAKO) according to the manufacturer's instructions, followed by counter-staining with hematoxylin (Merck).

Polymerase chain reaction (PCR)

Total RNA from CSP ($n = 3$ donors, passage three) was isolated as described[27] and RNA (5 μg) was reversely transcribed with the iScript cDNA Synthesis Kit according to the manufacturer's instructions (BioRad). The relative expression level of the housekeeping gene *glyceraldehyde-3-phosphate dehydrogenase* (*GAPDH*) was used to normalize samples. Real-time PCR using the i-Cycler PCR System (BioRad) was performed with 1 μl of cDNA sample using the SYBR Green PCR Core Kit (Applied Biosystems) in triplicates per donor ($n = 9$ assays in total). Relative quantitation of chemokine receptor expression (Table I) was performed and is given as percentage of the GAPDH product.

Statistical analysis

For analysis of chemokine levels in SF, the Kolmogorov–Smirnov method was applied for testing normal distribution of the data. For normal distributed data, the *t*-test was applied, while the Mann–Whitney rank sum test was used for data that failed normality testing. Differences were considered significant at $P < 0.05$.

For statistical analysis of CSP recruitment upon chemokine treatment, the Kolmogorov–Smirnov method was applied for testing normal distribution of the data. Normal distributed recruitment data were further analyzed with the one way repeated measures (RM) analysis of variance (ANOVA) and data without normal distribution with RM ANOVA on ranks. Subsequently, all pairwise multiple comparison procedures (Student-Newman–Keuls method (SNK) for all pairwise comparisons and Dunnett's method for comparison vs control/0 nM) were applied and differences were considered significant at $P < 0.05$. For calculation of exact *P*-values, the parametric paired *t*-test or the non-parametric Wilcoxon signed rank test was applied. The 95% confidence interval (CI) was calculated and the lower and upper limit is given.

Results

Chemokine levels in normal, RA and OA SF

To analyze chemokines in SF from normal [Fig. 1(A)], OA [Fig. 1(B)] and RA donors [Fig. 1(C)], human chemokine antibody arrays were used. The array configuration with all chemokines measured in duplicates is given in Fig. 1(D). Densitometric analysis showed that the chemokines CCL2, CCL24, CCL27 and CXCL5 are significantly reduced, while the chemokines CCL1, CCL7, CCL13 and CXCL1

Table I
Oligonucleotides

gene name	Reference sequence	Oligonucleotides (5′→3′) (up/down)	Product size (Base pairs)
GAPDH*	NM_002046	GGC GAT GCT GGC GCT GAG TAC/ TGG TCC ACA CCC ATG ACG A	149
CXCR1	NM_000634	CTG AGC CCC AAG TGG AAC GAG ACA/ GCA CGG AAC AGA AGC TTT ATT AGG A	152
CXCR2	NM_001557	CAA TGA ATG AAT GAA TGG CTA AG/ AAA GTT TTC AAG GTT CGT CCG TGT T	118
CXCR3	NM_001504	CCC GCA ACT GGT GCC GAG AAA G/ AGG CGC AAG AGC AGC ATC CAC AT	148
CXCR4	NM_003467	ATC CCT GCC CTC CTG CTG ACT ATT C/ GAG GGC CTT GCG CTT CTG GTG	231
CXCR7	NM_020311	GCT GCG TCA ACC CTG TCC TCT AC/ GGC CCA TCA CCC TGT TCA AAA A	224
CCR2	NM_000647	CTA CCT TCC AGT TCC TCA TTT TT/ ACA TTT ACA AGT TGC AGT TTT CAG C	100
CCR3	NM_178329	TTT GTC ATC ATG GCG GTG TTT TTC/ GGT TCA TGC AGC AGT GGG AGT AG	169
CCR4	NM_005508	GAG AAG AAG AAC AAG GCG GTG AAG A/ GGA TTA AGG CAG CAG TGA ACA AAA G	200
CCR9	NM_031200	TAT ACA GCC AAA TCA AGG AGG AAT C/ CAT GAC CAC GAA GGG AAG GAA G	137
CCR10	NM_016602	GGG CTG GAG TCT GGG AAG TGC/ ACG ATG ACG GAG ACC AAG TGT GC	183
XCR1	NM_005283	CAT CAT GAC CAT CCA CCG CTA CC/ TCG AGG ATG GAG GAC AGG ATG C	129

* Glyceraldehyde-3-phosphate dehydrogenase.

Table II
Profile of selected chemokines in SF derived from normal, OA and RA donors

Chemokine		ND		OA		RA	
Name	Synonym	Mean	Range Max/Min	Mean	Range Max/Min	Mean	Range Max/Min
CXCL7	NAP2	48.5	89.9/5.8	36.3	60.6/23.0	39.1	65.3/23.9
CCL22	MDC	38.2	50.4/22.7	29.9	43.4/21.3	28.8	31.0/24.7
CCL2	MCP1	26.4	48.0/14.7	12.1	28.5/1.1	9.9	14.4/5.9
CXCL5	ENA78	22.7	26.5/19.2	15.0	23.9/9.8	13.1	15.6/10.8
CXCL10	IP10	21.1	27.0/14.0	16.7	23.2/9.4	16.2	20.5/14.2
XCL1	Lymphotactin	20.4	29.1/16.7	15.5	22.2/5.1	15.5	20.1/13.2
CCL24	Eotaxin2	20.4	28.5/10.9	12.1	29.7/4.7	10.2	13.5/8.3
CCL27	CTACK	16.7	22.1/13.8	10.3	21.0/3.4	8.9	10.7/8.3
CXCL12	SDF1α	2.3	9.6/0.0	1.1	3.6/0.0	0.7	2.3/0.0
CCL25	TECK	1.8	5.7/0.2	3.6	9.5/0.0	2.3	4.9/0.1

Chemokine		Ratio			*P*-value		
Name	Synonym	OA/ND	RA/ND	OA/RA	OA/ND	RA/ND	OA/RA
CXCL7	NAP2	−1.3	−1.2	−1.1	–	–	–
CCL22	MDC	−1.3	−1.3	1.0	–	–	–
CCL2	MCP1	−2.2	−2.7	1.2	–	0.0481	–
CXCL5	ENA78	−1.5	−1.7	1.1	0.0241	0.0002	–
CXCL10	IP10	−1.3	−1.3	1.0	–	–	–
XCL1	Lymphotactin	−1.3	−1.3	1.0	–	–	–
CCL24	Eotaxin2	−1.7	−2.0	1.2	–	0.0102	–
CCL27	CTACK	−1.6	−1.9	1.1	–	0.0079	–
CXCL12	SDF1α	−2.1	−3.3	1.6	–	–	–
CCL25	TECK	2.0	1.3	1.6	–	–	–

showed elevated levels in RA SF compared to ND SF. In OA SF, CXCL5 was significantly reduced compared to normal SF and the level of CCL1 was decreased in OA SF compared to RA SF (Table I, Supplementary material). The highest spot intensities were found for the chemokines CXCL7 and CCL22 with 28–48% of the intensity measured in positive control spots, while the lowest intensities were found for CCL7 and CCL8, which were virtually absent in normal SF. The complete chemokine profile is given in Table II of the Supplemental material.

Since a previous study showed that RA SF showed reduced chemotactic activity on human subchondral progenitors compared to normal and OA SF, the chemokines CCL2, CCL24, CCL27 and CXCL5, which are significantly reduced in RA SF, were selected for further analysis in chemotaxis assays. In addition, representative chemokines were selected that showed high (CXCL7, CCL22), moderate (CXCL10, XCL1) or low (CXCL12, CCL25) intensities in normal, OA and RA SF (Table II).

Chemokine induced migration of human subchondral progenitor cells

Stimulation of CSP with CCL2, CCL24 or CXCL7 showed no effect on progenitor migration (data not shown). The chemokines CCL22, CCL27, CXCL5 and CXCL12 did not recruit CSP but showed a moderate inhibition of cell migration (Table III). In controls, in the absence of CCL22, spontaneous migration occurred with a mean migration index of 1.0 and a CI of 0.81–1.19. Stimulation with 1,000 nM of CCL22 reduced the mean migration index (CI ranging from 0.42 to 0.80) compared to untreated controls. Compared to untreated CSP, 250 nM–500 nM CCL27 showed an inhibitory effect on the migration of cells. In addition, stimulation with 250 nM of CCL27 significantly reduced the migration index (mean 0.54) compared to all other doses tested (mean index ranging from 0.72 to 1.32). Significant inhibition of cell recruitment occurred in the presence of 250 nM and 1,000 nM CXCL5 (mean migration index of 0.47–0.55) as well as after stimulation with 50–500 nM CXCL12

Table III
Migration index (migrated CSP/control) of selected chemokines that showed an inhibitory effect on migration of subchondral progenitor cells

nM	N	Migration index			SNK $P<0.05$ vs 0 nM	*P*-value* vs 0 nM
		Mean	CI (95%) Lower limit	CI (95%) Upper limit		
CCL22						
0	9	1.00	0.81	1.19	–	–
1	9	1.22	0.61	1.84	No	0.4145
10	9	1.14	0.83	1.45	No	0.4170
50	9	0.91	0.70	1.13	No	0.4753
100	9	1.02	0.71	1.33	No	0.8720
250	9	1.03	0.84	1.22	No	0.7348
500	9	0.75	0.63	0.87	No	0.0240
1,000	9	0.61	0.42	0.80	No	0.0078
CCL27						
0	9	1.00	0.87	1.13	–	–
1	9	1.19	0.94	1.44	No	0.1731
10	9	1.32	1.02	1.62	No	0.0387
50	9	1.09	0.92	1.26	No	0.2855
100	9	0.81	0.69	0.93	No	0.0534
250	9	0.54	0.43	0.65	No	0.0008
500	9	0.72	0.52	0.92	No	0.0343
1,000	9	1.32	0.93	1.71	No	0.1151
CXCL5						
0	9	1.00	0.81	1.19	–	–
1	9	0.99	0.71	1.27	No	0.9414
10	9	0.74	0.54	0.94	No	0.0605
50	9	0.80	0.62	0.98	No	0.1568
100	9	0.81	0.59	1.03	No	0.2220
250	9	0.55	0.39	0.71	Yes	0.0043
500	9	0.47	0.34	0.60	Yes	0.0001
1,000	9	0.62	0.32	0.92	Yes	0.0534
CXCL12						
0	9	1.00	0.84	1.16	–	–
1	9	0.81	0.65	0.97	No	0.1322
10	9	0.89	0.71	1.07	No	0.3535
50	9	0.73	0.58	0.88	Yes	0.0019
100	9	0.50	0.31	0.69	Yes	0.0014
250	9	0.64	0.51	0.77	Yes	0.0039
500	9	0.40	0.19	0.61	Yes	0.0006
1,000	9	1.04	0.74	1.35	No	0.7207

* Paired *t*-test/Wilcoxon signed rank test.

Table IVa
Migration index (migrated CSP/control) of selected chemokines that showed stimulation of migration of subchondral progenitor cells

nM	N	Migration index			SNK $P < 0.05$ vs 0 nM	P-value* vs 0 nM
		Mean	CI (95%) Lower limit	CI (95%) Upper limit		
CCL25						
0	9	1.00	0.86	1.14	–	–
1	9	1.40	1.20	1.60	No	0.0124
10	9	1.37	1.17	1.57	No	0.0148
50	9	1.13	1.04	1.22	No	0.1641
100	9	1.07	0.96	1.12	No	0.4470
250	9	1.01	0.63	1.39	No	0.9696
500	9	1.91	1.06	2.76	No	0.0378
1,000	9	3.23	2.12	4.35	Yes	0.0018
CXCL10						
0	9	1.00	0.75	1.23	–	–
1	9	1.75	1.33	2.17	Yes	0.0061
10	9	2.31	0.91	3.71	Yes	0.0273
50	9	1.62	0.97	2.27	Yes	0.0758
100	9	2.00	1.23	2.77	Yes	0.0039
250	9	2.57	1.20	3.94	Yes	0.0291
500	9	2.56	1.72	3.41	Yes	0.0034
1,000	9	3.96	2.18	5.74	Yes	0.0039
XCL1						
0	9	1.00	0.87	1.13	–	–
1	9	1.22	1.04	1.41	No	0.0511
10	9	1.47	1.23	1.71	Yes	0.0015
50	9	1.30	0.96	1.64	No	0.0882
100	9	1.24	0.89	1.59	No	0.1308
250	9	1.01	0.80	1.22	No	0.9242
500	9	0.68	0.52	0.84	No	0.0072
1,000	9	0.84	0.49	1.19	No	0.2746

* Paired *t*-test/Wilcoxon signed rank test.

Table IVb
Number of migrated cells upon treatment of subchondral progenitor cells with selected chemokines

nM	N	Number of migrated cells			SNK $P < 0.05$ vs 0 nM	P-value* vs 0 nM
		Mean	CI (95%) Lower limit	CI (95%) Upper limit		
CCL25						
0	9	1,342	1,102	1,582	–	–
1	9	1,911	1,468	2,355	No	0.0156
10	9	1,846	1,479	2,213	No	0.0109
50	9	1,524	1,287	1,761	No	0.1641
100	9	1,451	1,154	1,748	No	0.3622
250	9	1,277	916	1,638	No	0.7585
500	9	2,435	1,553	3,317	No	0.0308
1,000	9	4,161	2,950	5,372	Yes	0.0009
CXCL10						
0	9	1,331	766	1,896	–	–
1	9	2,155	1,299	3,011	Yes	0.0315
10	9	2,102	1,766	2,438	Yes	0.0243
50	9	1,843	1,156	2,530	No	0.0947
100	9	2,123	1,629	2,617	Yes	0.0008
250	9	2,444	1,953	2,935	Yes	0.0080
500	9	2,837	2,078	3,596	Yes	0.0007
1,000	9	4,277	3,316	5,238	Yes	<0.0001
XCL1						
0	9	1,907	1,240	2,574	–	–
1	9	2,302	1,474	3,130	No	0.0492
10	9	2,726	1,727	3,726	Yes	0.0046
50	9	2,596	1,319	3,874	No	0.0913
100	9	2,537	1,283	3,791	No	0.1017
250	9	1,991	1,090	2,892	No	0.7080
500	9	1,327	692	1,962	No	0.0096
1,000	9	1,406	753	2,059	No	0.1339

* Paired *t*-test/Wilcoxon signed rank test.

(mean migration index of 0.40–0.73) compared to untreated controls.

In contrast, the chemokines CCL25, CXCL10 and XCL1 significantly recruited human subchondral mesenchymal progenitors (Table IV). CSP cells stimulated with 1,000 nM CCL25 or 1 nM–1,000 nM CXCL10 as well as 10 nM XCL1 showed significantly elevated migration indices compared to CSP not treated with a chemokine (Table IVa). Stimulation of CSP with 1,000 nM CCL25 (mean migration index of 3.23) or 1,000 nM CXCL10 (mean migration index of 3.96) showed effective cell recruitment and significantly increased CSP migration compared to CSP stimulated with 0 nM–500 nM of the chemokine. Compared to 0 nM, 1 nM and 50–1,000 nM XCL1, 10 nM of XCL1 showed significant cell recruitment with the highest migration index (mean migration index of 1.47). The use of 500 nM and 1,000 nM of XCL1 inhibited CSP migration compared to untreated cells, while 500 nM XCL1 reduced the number of migrating cells compared to all other doses tested.

Numbers of migrating CSP upon chemokine treatment are given in Table IVb. Compared to controls (mean; 1,342 CSP), stimulation of CSP with 1,000 nM CCL25 (mean; 4,161 CSP) significantly increased the number of migrating cells. In cell recruitment by CXCL10 and significantly elevated compared to controls, the highest numbers of migrating CSP occurred in the presence of 500 nM (mean; 2,837 migrating CSP) and of 1,000 nM CXCL10 (mean; 4,277 migrating CSP). In the presence of 10 nM XCL1 in mean 2,726 CSP migrated.

Chemokine receptors in subchondral progenitors

To assess whether human subchondral progenitors have chemokine receptors for chemokines tested in this study, PCR analysis was performed (Fig. 2). In general, CSP showed low levels of the particular chemokine receptors. CSP showed low expression levels of CXCR1-4 and CXCR7 (mean, 0.0004–0.2451% of the expression level of GAPDH) as well as of CCR2-3 and CCR9-10 (mean, 0.009–0.141%). CCR4 was virtually not expressed. The XCL1 receptor XCR1 showed a mean expression level of 0.06% of the expression level of GAPDH. For the CCL25 receptor CCR9, the mean expression level was 0.03% of the level found for GAPDH, while the CXCL10 receptor CXCR3 showed a level of 0.2451% of the expression level of GAPDH.

Immuno-histochemical staining (Fig. 3) confirmed the presence of the CXCL10 receptor CXCR3, the CCL25 receptor CCR9 and the XCL1 receptor XCR1. Controls that were incubated with IgG and the detection system gave no signal and indicated specificity of the antibody staining.

Discussion

In this study, we showed that human SF derived from normal, OA and RA donors contain different levels of distinct chemokines. A candidate subset is presented that shows chemokines with reduced levels in RA or OA compared to normal SF or robustly high levels in all SF. The chemokines CCL22, CCL27, CXCL5 and CXCL12 inhibited the migration of human subchondral mesenchymal progenitor cells *in vitro*, while CCL2, CCL24 and CXCL7 had no effect on progenitor cell attraction. The candidate chemokines CCL25 (TECK), CXCL10 (IP10) and XCL1 (Lymphotactin) effectively recruited human subchondral progenitors. This suggests that SF contains chemokines that may contribute to the migratory effect of SF on human subchondral progenitors in microfracture.

The microfracture technique induces the formation of a cartilaginous repair tissue by introducing multiple perforations into the subchondral bone that allow for accessing the bone marrow and

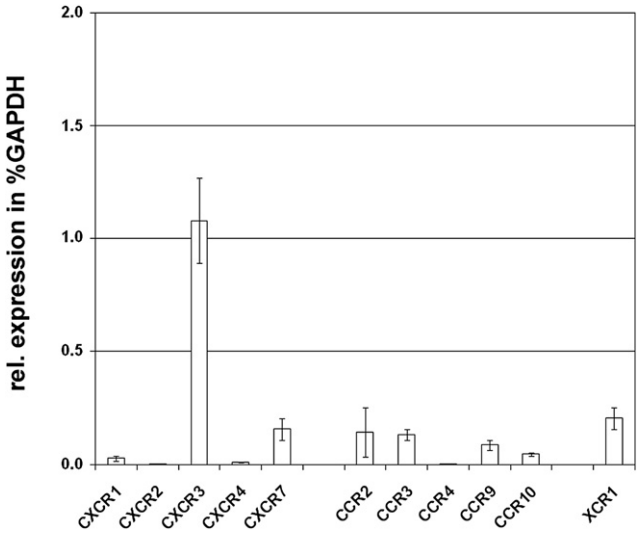

Fig. 2. Gene expression analysis of human progenitor cells for selected chemokine receptors. Real-time PCR analysis ($n = 9$) of three independent CSP preparations (passage three) showed a low level of expression of CXCR1-4 and CXCR7 as well as CCR2, CCR3, CCR9, CCR10 and XCR1. CCR4 was not detected. The mean of each expression level is plotted and the error bars represent 95% CI (lower/upper limit).

bleeding into the defect. Thus, multi-potent progenitor cells populate the defect and form the cartilage repair tissue[28]. The mechanisms that underlie the migration of subchondral progenitor cells from the marrow into the defect as well as their subsequent differentiation towards chondrocytic cells still remain sparsely understood. Migration and differentiation of the progenitors may occur passively or may be actively triggered and influenced by factors from the bone or the SF. Particular growth and differentiation factors as well as cytokines enter the defect with the blood flow or may be released by the subchondral bone. These factors may be involved in the recruitment and subsequent chondrogenic differentiation of mesenchymal progenitors[4,7]. Recently, we have shown that mesenchymal progenitor cells from the subchondral bone have a high chondrogenic potential and that the chondrogenic developmental process may be negatively influenced by inflammatory SF conditions found in RA[24,29]. In addition, SF actively recruits mesenchymal progenitor cells from bone marrow[10] or potentially from the synovial tissue[30]. As shown here, the candidate chemokines CCL25, CXCL10 and XCL1 that were deduced from the different chemokine compositions of SF from normal, OA and RA donors significantly recruit human subchondral mesenchymal progenitors, while others have no or an inhibitory effect on cell migration. In MSC derived from, for instance, iliac crest bone marrow aspirates, the chemokines CXCL5, CXCL7, CXCL10, CCL22, and CCL25 have been shown to induce cell migration[31–35]. The migratory effect of CXCL12 on MSC is discussed controversially with studies showing CXCL12 mediated migration of MSC, while others found no effect[21–23]. In contrast, the chemokines CCL2, CCL24, CCL27 and XCL1 are known for not recruiting human MSC[23,31,36]. The variable effects of chemokines on mesenchymal cells may due to the restricted and partly different set of chemokine receptors found in cells of different types or origin as known from stem cells derived from iliac crest, periosteal cells or stem cells from osteoarthritic femoral heads[23,37,38]. Although mesenchymal progenitors derived from the subchondral bone marrow share a variety of characteristics with MSC derived from bone marrow aspirates, like the cell-surface antigen pattern and the high chondrogenic differentiation capacity[24], there are obviously differences in the potential to migrate and/or in the subset of chemokines that stimulate migration of the progenitors. As shown here, subchondral mesenchymal progenitors do express chemokine receptors, are recruited by a subset of chemokines that is present in human SF and therefore chemokines may play an important role in the migration of subchondral stem cells in microfracture treatment. However, further studies have to elucidate which particular chemokine may mediate migration of mesenchymal progenitors *in vivo* and/or in

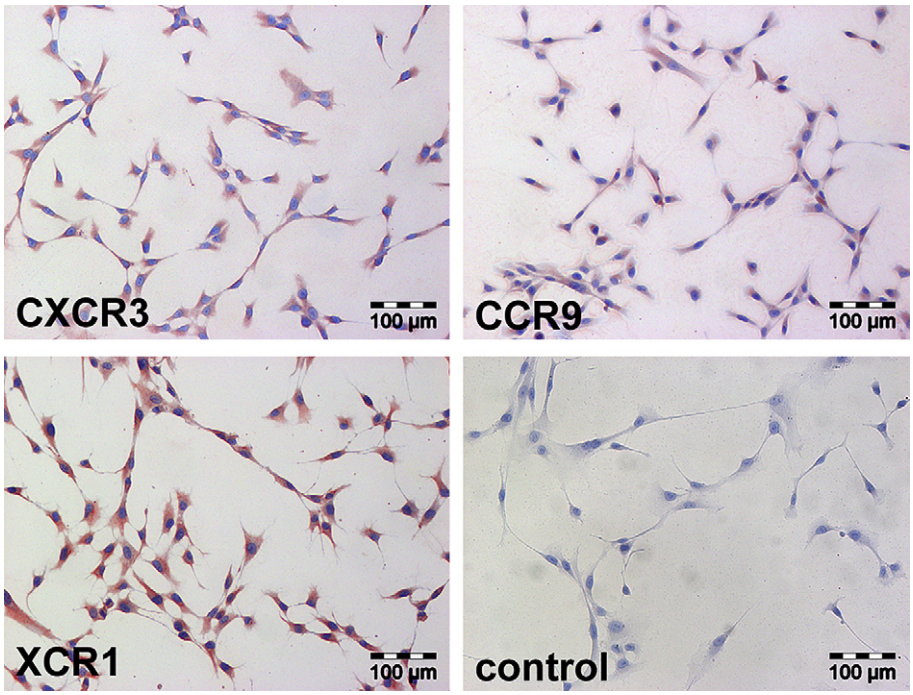

Fig. 3. Immuno-histochemical staining of chemokine receptors on human progenitor cells. Immuno-histochemical staining of CXCR3, CCR9 and XCR1 showed presence of the respective receptors on human subchondral progenitor cells on the protein level. Controls gave no signal and show specificity of the antibody staining.

microfracture. Further limitations of this *in vitro* study are that it is not clear whether the chemokines detected in the SF are biologically active or inactive due to degradation or partial degradation. The analysis of chemotactic events does not take into account that growth factors from blood and from the subchondral bone as well as from the synovial tissue may influence the migration potential of mesenchymal progenitors in microfracture. In addition, in the joint, potential chemotactic chemokines are present in mixture with other chemokines as well as with growth factors and proteinases. Therefore, to resemble the natural situation more closely, further studies are needed that analyze potential synergistic or antagonistic effects of chemokine mixtures on stem cell migration in microfracture. As shown here in subchondral CSP cells and in line with previous studies with human MSC and periosteal cells[23,35,38], the doses of chemokines that stimulated or inhibited cell migration were high and not physiological. Stem cells, periosteal cells and CSP cells express a variety of chemokine receptors at a low level, this may explain the high doses of chemokines needed for effective CSP recruitment or inhibition of migration. In addition, the protein content of SF was normalized prior to measurement of the chemokine levels, with higher protein content in RA and OA compared to ND. Interestingly, chemokine levels found in RA and/or OA were partly reduced compared to ND. This is in contrast to a variety of studies that show that chemokines increase in the diseased condition[14]. However, in general, in these studies the protein content was not normalized. Although highly speculative, this may indicate that in the diseased condition, (1) the protein content and chemokines may increase in general due to invasion of cells into the synovial lining layer and into the SF and (2) particular chemokines may be disease-specifically induced in distinct cell type like monocytes or T-cells. Clearly, further studies are needed that may elucidate the general or disease-specific increase in joint diseases. Although the synovial samples used in this study may be epidemiologically representative for the diseased joint conditions, a limitation of the study is that the samples derived from the normal, RA and OA donors are in-homogenous with respect to gender, age and potentially age-related hormonal changes in the menopause as well as their disease-specific treatment regimen. However, correlation of the obtained profiles to a particular treatment, disease activity/severity or age was not evident. Since the number of samples used in this study is limited, further studies are needed that address the impact of age, hormones and medicinal drugs on the profile of chemokines in SF.

From the clinical point of view, the influence of SF or components of the fluid like chemokines on cartilage repair by microfracture remains unclear and further studies are needed. Since the microfracture technique often results in a hyaline to fibrous cartilage repair tissue with a limited short-term durability, approaches are demanded that may enhance the content of matrix components and improve cartilaginous repair tissue formation in microfracture[5]. In addition, enhancement of stem cell migration into and enrichment of multi-potent progenitors within the defect, which subsequently differentiate along the chondrogenic lineage and form the repair tissue, may be also a promising approach for the improvement of cartilage repair in microfracture. Recently, it has been shown that MSC from bleeding microfractures enter a collagen membrane used for covering of the defect[39] and covering of microfractured defects with a collagen matrix, fibrin glue and autologous serum is suggested to be a promising treatment option for cartilage defects[40]. In a rabbit model, cartilage repair has been shown by mesenchymal progenitor cells from bone marrow that were attracted by a serum containing blood clot in combination with a chitosan scaffold[41,42]. In addition, the benefit of covering microfractured cartilage defects has been proven recently in the ovine model. Compared to conventional microfracture treatment, the covering of microfractured defects with a resorbable scaffold made of polyglycolic acid and hyaluronan that was immersed in autologous serum for cell recruitment improved the formation of cartilage repair tissue that was rich in type II collagen[43,44]. The first clinical applications of the polyglycolic acid/hyaluronan scaffold fixed with resorbable pins in traumatic and degenerated cartilage defects showed excellent defect filling as assessed by magnet resonance imaging after 1 year follow-up[45]. Although highly speculative, particular chemokines like CCL25, CXCL10 and XCL1 as well as/or additional components of the SF like lysophosphatic acid[46] may have an impact in the population of cartilage defects by multi-potent progenitors and may therefore candidates for the improvement of the microfracture technique. However, further studies are needed to clarify the role of chemokines in stem cell migration and their possible use for improving cartilage repair techniques like microfracturing.

Finally, our report shows the chemokine profile of normal and arthritic human SF and suggests that a subset of these chemokines may contribute to the recruitment of human mesenchymal progenitors from the subchondral bone.

Contributions

ME, GK and UF performed the chemotaxis assays, the statistical analysis of data and analyzed the gene expression data. KA and ME performed the chemokine array analysis. KN carried out the chemokine receptor staining. JR and MS contributed to the conception and design of study. TH collected SF and corresponding clinical data. CK designed the study and drafted manuscript. All authors revised the manuscript critically and finally approved it.

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Conflict of interest

GK, UF, ME, KN and CK are employees of TransTissue Technologies GmbH (TTT), a subsidiary of BioTissue Technologies GmbH (BTT). TTT develops products and cartilage treatment strategies in the field of regenerative medicine. MS works as a consultant for BTT. All other authors declare no conflict of interests.

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Supplementary material

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.joca.2010.08.003.

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