Synovial Fluid Recruits Human Mesenchymal Progenitors from Subchondral Spongyous Bone Marrow

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ABSTRACT: Microfracture is a frequently used reparative technique that induces a healing response in articular cartilage defects. Penetration of the subchondral bone leads to blood clot formation, allows multipotent mesenchymal cells to access the defect and, subsequently, leads to cartilaginous repair tissue. The aim of our study was to analyze the chemotactic recruitment of human subchondral spongyous bone marrow-derived cells by synovial fluid (SF) from normal donors (ND), patients with osteoarthritis (OA) and rheumatoid arthritis (RA). Subchondral spongyous bone marrow-derived mesenchymal progenitors were isolated from bone cylinders after high tibial osteotomy and analyzed for the presence of stem cell-related cell surface antigens by flowcytometry. Recruitmen of subchondral progenitors by normal SF and SF from donors with degenerated joint diseases was documented by using a modified Boyden chamber chemotaxis assay. The chemotaxis assay demonstrated that synovial fluid has the potential to recruit mesenchymal progenitors in vitro. SF from normal donors and patients with OA showed no difference in the potential to stimulate cell migration. SF obtained from RA donors showed significantly reduced cell recruitment compared to SF derived from OA patients (p = 0.0054) and normal donors (p < 0.0001). The chemotactic activity of SF obtained from normal donors and from patients with degenerative joint diseases suggests that SF may be actively involved in the migration of progenitors in cartilage defects after microfracture. © 2007 Orthopaedic Research Society. Published by Wiley Periodicals, Inc. J Orthop Res 25:1299–1307, 2007

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INTRODUCTION

Articular cartilage has a limited healing capacity. Therefore, current orthopaedic and surgical interventions aim to repair cartilage lesions and to restore articular cartilage surfaces. Techniques for the repair and restoration of articular cartilage comprise procedures like shaving, debridement, OATS (Osteochondral Autograft Transfer System), ACT (Autologous Chondrocyte Transplantation), and bone marrow stimulating techniques such as microfracture or drilling. Microfracture is a frequently used reparative technique that induces a healing response by stimulating the subchondral bone marrow in areas of articular cartilage damage. The microfracture technique is described by Steadman and colleagues in detail. In brief, all loose or marginally attached cartilage around the defect has to be debrided leaving healthy well-attached viable cartilage. Excessive damage to the subchondral bone should be avoided and the debridement has to respect the subchondral plate while the calcified cartilage layer is removed. Subsequently, the access to blood, blood-derived cells, as well as to bone marrow-derived mesenchymal progenitors is achieved by creating multiple holes or microfractures in the exposed subchondral bone plate adjacent to the healthy cartilage rim and in the center of the defect. Penetration of the subchondral bone leads to blood clot formation in

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the defect, stimulates the marrow, and allows multipotent mesenchymal cells and growth factors from the marrow to access the defect. The bone marrow stimulating techniques are considered to be the least invasive treatments, are relatively inexpensive, can be performed arthroscopically, and are recommended for smaller chondral lesions without bone involvement. Marrow stimulating techniques promote the formation of hyaline to fibrous cartilaginous repair tissue that may be biomechanically inadequate in the long term. Animal studies in horses demonstrated that treatment of full-thickness chondral defects with microfracture increases repair tissue formation and the percentage of type II collagen in the defects compared to nontreated defects. However, the nature of the invasion by means of active recruitment or passive influx of mesenchymal progenitor cells, factors that may actively recruit precursors to the defects, and the impact of degenerative joint disease conditions like rheumatoid arthritis and osteoarthritis on the recruitment of mesenchymal progenitors have still to be elucidated.

The microfracture technique is accompanied by a specific and guided ingrowth of cartilage forming cells, rather than by the migration of an unselected population of mesenchymal stem cells. Recently, a variety of chemokines like CXCL12 (SDF-1α), CXCL13 (BCA-1/BLC), CXCL16, CCL5 (RANTES), CCL19 (MIP-3β), and CCL25 (TECK), as well as human plasma, platelet-derived growth factor (PDGF-bb), hepatocyte growth factor (HGF), and bone morphogenetic proteins as members of the transforming growth factor (TGF)-β superfamily of growth and differentiation factors have been shown to chemotactically guide mesenchymal stem cells derived from bone marrow in cell culture experiments. Synovial fluid nourishes articular cartilage, lubricates articular joint surfaces, contains hyaluronic acid, growth factors like TGF-β family members and chemokines, and induces chondrogenic lineage development of bone marrow-derived mesenchymal stem cells. Since synovial fluid may contain putative chemotactic agents and its composition is altered in joint diseases like RA and OA, we addressed the question whether synovial fluid has the capacity to actively recruit mesenchymal progenitors from the subchondral spousous bone marrow and to which extent recruitment is impaired in degenerated joint diseases.

Therefore, we hypothesize that synovial fluid from normal, OA, and RA donors recruit human mesenchymal progenitors derived from subchondral spongy bone marrow, and that the potential of synovial fluid to recruit mesenchymal precursor cells is reduced in diseased joint conditions.

**MATERIALS AND METHODS**

**Isolation and Culture of Human Mesenchymal Progenitors from Subchondral Spongy Bone Marrow**

Human cortico-spongy bone (two male, one female; age 40–62 years) was obtained from the lateral tibia head during high tibial closed wedge osteotomy. The distance from the harvest side to the subchondral bone joint line was approximately 4 cm. To isolate progenitor cells, bone cylinders were derived from the excised bone and washed with Hank's Salt Solution (Biochrom AG, Berlin, Germany), separated from connective tissue, and cut into small pieces. Bone fragments were digested for 4 h in OptiPro SFM medium (Invitrogen Karlsruhe, Germany), 5% human Serum (German Red Cross, Berlin, Germany), 100 U/ml penicillin (Biochrom), 100 mg/ml streptomycin (Biochrom), and 256 U/ml collagenase XI (Sigma-Aldrich, Munich, Germany) in a spinner flask (Weaton Millville, NJ) under gentle stirring at 37°C.

After discarding the supernatant and washing with Hank's Salt Solution, the bone fragments were placed into Primaria cell culture flasks (Becton Dickinson, Heidelberg, Germany) and cultured in OptiPro SFM medium supplemented with 5% human serum, 100 U/ml penicillin (Biochrom), 100 mg/ml streptomycin (Biochrom), and 4 mM L-glutamin (Biochrom) under standard cell culture conditions. After 5 to 7 days, first cells were evident. After reaching 70% confluency, cells were trypsinized with trypsin/EDTA solution (Biochrom) and subcultured at a density of 8 × 10^5 cells/cm^2.

**FACS Analysis of Typical MSC Cell Surface Antigens**

The subchondral spongy bone marrow-derived cells (passage 3) were characterized by flowcytometric analysis (FACS). A single cell suspension of 2.5 × 10^5 cells was washed once with PBS containing 0.5% BSA and incubated with biotin-labeled mouse anti-human SH-2 (CD105), fluorescein isothiocyanate (FITC) labeled mouse anti-human CD45 (German Rheumatism Research Center, Berlin, Germany), FITC labeled mouse anti-human CD44 and CD90, R-phycocerythrin (PE) labeled mouse anti-human SH-3 (CD73), as well as PE labeled CD14, CD166, and CD34 (Becton Dickinson) for 15 min on ice. For staining, biotin-labeled SH-2 was incubated with streptavidin coupled with allophycocyanin (APC) for 10 min on ice, washed with PBS/BSA and finally incubated with CD73-PE antibody. Staining of all other antibodies was performed in a single step. Finally, cell samples were washed and prior to the analysis with the FACSCalibur (Becton Dickinson), cells were stained with propidium iodide (Sigma) to detect and exclude dead cells. Data
were evaluated using the CellQuest software (Becton Dickinson).

Collection of Synovial Fluid

RA-synovial fluid was obtained by joint puncture from five female donors (mean age 45.4 years; mean DAS 28 6.6; mean ESR (1 h) 74.2, and mean CrP 6.96 mg/ml) that showed reliable rheumatoid arthritis according to the revised American College of Rheumatology criteria for the Classification of Rheumatoid Arthritis. Two donors with rheumatoid arthritis received disease modifying anti-rheumatic drugs (DMARD) and steroidal anti-inflammatory drugs (SAID), one donor had SAID and nonsteroidal anti-inflammatory drugs (NSAID), one donor had DMARD, and one donor received only NSAID. None of the patients received intraarticular therapy. All patients had an active disease with a high DAS 28 and were punctured because of knee effusion.

OA-synovial fluid was obtained by joint puncture from five female donors (mean age 62.0 years) that showed reliable osteoarthritis according to the American College of Rheumatology criteria for the classification and reporting of osteoarthritis of the knee. Three of the donors with osteoarthritis received NSAID and two donors had no medicaments at the time of joint puncture. ND-synovial fluid was obtained postmortem from five organ donors (three female, two male; mean age 53.2 years) without joint diseases. All procedures were performed in consent with the ethical committee of the Charité-Universitätsmedizin Berlin.

All synovial fluids were carried on ice and centrifuged after collection to remove cells. Finally, synovial fluid was stored at −80°C.

Cell Migration Assay

For the cell migration assay, the ChemoTX® Assay System (Neuroprobe) with a polycarbonate membrane with a pore size of 8 μm and a pore density of 1 × 10⁹ pores/mm² was used according to the manufacturer’s recommendations (ChemoTX® System protocol, Neuroprobe).

In brief, synovial fluids of individual donors (ND, n = 5; RA, n = 5; OA, n = 5) were diluted in DMEM low glucose medium containing 1 × ITS (Sigma), 100 U/ml penicillin, and 100 mg/ml streptomycin to a final concentration of 50% synovial fluid in medium and given into the lower wells of the microplate in triplicate. DMEM supplemented as described but without synovial fluid served as a negative control. DMEM low glucose medium containing 10% FBS, 100 U/ml penicillin, and 100 mg/ml streptomycin was used as a positive control. Mesenchymal progenitor cells (passage 3) derived from independent donors (n = 3) were trypsinized, washed once in DMEM low glucose medium containing 1 × ITS, 100 U/ml penicillin, and 100 mg/ml streptomycin, and 3 × 10⁴ cells per well (n = 3) were pipetted onto the membrane in the upper well. The ChemoTX® microplate was incubated at 37°C, 5% CO₂, and humidified air. After 20 h, the cells attached to the filter membrane were fixed with acetone/methanol 1:1 (v:v) for 3 min. After fixation, residual cells on top of the membrane were gently wiped off with common cotton swaps. Afterwards, the membrane was rapidly stained with Hemacolor for microscopy (Hemacolor rapid stain; Merck). Cells that migrated through the filter pores and attached underneath the membrane were analyzed by microscopy. For standardization of the assay, photographs were taken representing the left, the middle, and the right areas of the well. The cells in these representative visual fields were counted and the number of migrated cells was extrapolated to the total migration area.

Since progenitor cells spontaneously migrate through the membrane, the cell numbers determined in the negative control without chemotactic agents were subtracted from the cell numbers counted after stimulation of the cells with a putative chemotactic agent. In total, for the cell migration assay, we used three individual progenitor cell preparations stimulated each with n = 5 synovial fluids from OA donors, n = 5 synovial fluids from RA donors, and n = 5 synovial fluids from normal donors. All assays were performed in triplicate.

Statistical Analysis

For statistical analysis, the nonparametric Mann–Whitney rank sum test was applied and differences were considered significant at p < 0.05.

RESULTS

Morphology and MSC-Related Cell Surface Antigen Pattern of Subchondral Spongy Bone-Derived Progenitors

Using human serum as medium supplement, the outgrowth of human subchondral spongy bone marrow-derived cells started within 7 days. The cells adhered to the cell culture surface (Fig. 1A) and formed islands surrounding the bone fragment. After subculturing of the cells, we obtained a single cell suspension whose cells adhered rapidly to the tissue culture plastic surface and grew in monolayer culture. These small cells showed a stable fibroblast-shaped morphology over at least 5 passages (Fig. 1B).

To characterize the cells derived from the spongy bone, the antigen pattern of typical cell surface markers known from bone marrow-derived mesenchymal stem cells was determined by flowcytometric analysis (Fig. 2). The cells were negative for the early hematopoietic stem cell marker CD34, the lipopolysaccharide receptor CD14, and the leucocyte antigen CD45. In contrast, the cells presented the hyaluronic acid receptor CD44, ecto-5'-nucleotidase CD73 (SH-3), thy-1 cell surface antigen CD90, endoglin receptor CD105 (SH-2), and the activated leucocyte cell adhesion
molecule CD166 (ALCAM). The cell population showed variable presentation of CD166 and CD44, with 85% CD166 and 66% CD44 positive cells. The progenitors were homogenously positive for SH-2/CD73 double staining (99%) and CD90 (96%).

**Active Cell Recruitment**

For determining the chemotactic activity of synovial fluid (Fig. 3), cells (passage 3) were seeded onto the polycarbonate membrane of the ChemoTX® Assay System. Here the cells adhered to the membrane within 20 h and various cells started to migrate through the pores of the membrane, when synovial fluid was used as a chemoattractant. The migrated cells firmly attached to the lower surface of the membrane. Hemacolor staining of these cells showed bright pink nuclei with light blue cytoplasm (Fig. 3A). In contrast, the cells did not migrate when synovial fluid was absent (Fig. 3B), and only a few cells were detected at the bottom of the polycarbonate membrane. The number of human subchondral spongy bone marrow-derived cells that migrated towards a serum-reduced medium containing either 50% synovial fluid obtained from RA, OA, or ND donors are shown in Figure 4. Normal synovial fluid reproducibly recruited between 2,800 and 16,000 mesenchymal progenitors. Synovial fluid obtained from osteoarthritis donors recruited between 4,000 and 16,000 cells with one synovial fluid sample that reproducibly failed to induce the migration of one of the mesenchymal progenitor cell preparations. In contrast, RA-synovial fluid showed a reduced chemotactic activity and stimulated between 2,000 and 12,000 cells to migrate. In addition, one synovial fluid sample (SF-3) did not recruit mesenchymal cells at all, and the sample SF-1 showed no chemotactic activity on cells derived from one of the progenitor cell preparations. In the negative control, containing DME medium without synovial fluid, 490 cells ± 158 cells migrated.

Statistical analysis (Fig. 5) of the differences regarding the chemotactic recruiting potential of synovial fluid on mesenchymal progenitors from subchondral spongy bone marrow confirmed that synovial fluid obtained from OA donors recruited as many progenitors as normal synovial fluid ($p = 0.4076$). RA-synovial fluid showed a significantly reduced potential to recruit progenitors compared to normal ($p < 0.0001$) and OA ($p = 0.0054$) synovial fluid.

**DISCUSSION**

In the present study, we showed that synovial fluid recruits mesenchymal progenitor cells derived from the subchondral spongy bone marrow. Compared to synovial fluid from normal or OA donors, the potential of synovial fluid for cell recruitment in a modified Boyden chamber assay was impaired when synovial fluid of RA patients was used. In contrast, synovial fluid of OA patients did not alter cell migration compared to normal donors. In addition, the subchondral spongy bone marrow-derived mesenchymal progenitor cells presented a cell surface antigen pattern that is similar to the pattern known from bone marrow-derived mesenchymal stem cells (MSC).

In microfracture or Pridie-drilling, blood escaping from the damaged bone blood vessels forms a hematoma that temporarily fills the injury site.
Fibrin forms within the hematoma and platelets bind to fibrillar collagen. A continuous fibrin clot fills the bone defect and extends for a variable distance into the cartilage defect. Platelets within the clot release vasoactive mediators and growth factors or cytokines, which influence multiple cell functions including migration, proliferation, differentiation, and matrix synthesis,\(^3\) including factors of the TGF-beta family and platelet-derived growth factor (PDGF). Bone matrix also contains growth factors such as factors of the TGF-beta family, bone morphogenetic proteins (BMPs), PDGF, insulin-like growth factors (IGF), and possibly others.\(^4\) Release of these growth factors may have an important role in the repair of osteochondral defects. In particular, they probably stimulate vascular invasion and migration of undifferentiated cells into the clot, and influence the proliferative and synthetic activities of the cells.\(^5\) These factors may initiate or promote the healing sequence of cartilage defects after microfracture and may induce migration of cells into the defect and subsequent formation of repair tissue. After entering the defect, the undifferentiated mesenchymal cells proliferate, take the round shape of chondrocytes and synthesize a new matrix containing type II collagen. These cells produce regions of hyaline-like cartilage in the chondral and bony portions of the defect. Six to eight weeks after injury, the repair tissue within the chondral region of osteochondral defects contains many chondrocyte-like cells in a matrix consisting of type II collagen, proteoglycans, some type I collagen, and noncollagenous proteins.\(^6\)

In this context, the role of synovial fluid as an important component of the articular environment in cartilage repair and microfracture is still unclear. Synovial fluid has been shown to promote cartilage formation of bone marrow-derived mesenchymal stem cells; hyaluronic acid, as the most abundant component in synovial fluid, supported chondrogenic differentiation and matrix formation of MSC in tissue engineering.\(^13,22\) Also, synovial fluid plays an important role for cartilage nutrition and moreover for regeneration after damage of the subchondral bone.\(^23\) As shown here, synovial fluid also may play a role in the recruitment of progenitors that subsequently form repair tissue. In inflammatory diseases with secondary degenerative changes like RA, the composition of synovial fluid is altered and the chemokine cocktail differs from normal donors. Since cytokines and
diseases have affected the expression or levels of growth factors that may contribute to cell migration. The analysis of BMP levels and localization of BMP expression in synovial tissues from normal, OA, and RA donors demonstrated that the expression of the BMPs 4 and 5 is reduced in RA and OA compared to normal synovial tissues.\textsuperscript{29} Ertenli and colleagues showed that the level of factors of the TGF-beta family is higher in RA-synovial fluid than in fluid derived from OA donors.\textsuperscript{30} As a result, synovial fluid does not only nourish the cartilage but may also play an important role in active cell recruitment.

Obviously, synovial fluid contains a variety of growth and chemotactic factors, for example, factors of the CCL and CXCL chemokine family, as well as factors of the TGF-beta family that have been shown to recruit cells in a dose-dependent manner.\textsuperscript{8,31,32} Here we show that the number of mesenchymal progenitor cells, which are recruited by synovial fluid from RA patients, is significantly lower than from OA patients or normal donors. Therefore, these factors may contribute to migration of progenitors during microfracture. Composition of synovial fluid is altered, dependent on the disease, that may lead to the reduced recruitment capacity of RA-synovial fluid due to enhanced or reduced amounts of chemokines or growth factors.

There might be a two-step procedure for multipotent cells migrating into the defect site. In a first step, mesenchymal multipotent cells could be flushed in a passive process through the drill holes into the defect by the blood flow. In a second step, cells may be recruited into the defect by an active process by chemotactic active substances.

Here we show that synovial fluid derived from normal and diseased donors induced the recruitment of mesenchymal progenitors to a different extent. However, limitations of our study may be that progenitors were derived from donors with OA and therefore may not represent normal mesenchymal precursor cells. Murphy and colleagues reported that mesenchymal stem cells derived from bone marrow of OA donors have a reduced proliferation capacity and show a weaker in vitro chondrogenic and adipogenic differentiation potential compared to normal stem cells. The osteogenic differentiation capacity was not affected.\textsuperscript{33} However, it is still unclear whether osteoarthritis or other joint diseases have an impact on the migration ability of mesenchymal progenitors. Additionally, this in vitro recruitment system does not take into account the possibility that potential signals from the underlying bone, the synovial tissue, or other cells (e.g., dendritic cells) might influence the

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**Figure 3.** Hemacolor staining of a polycarbonate membrane of the ChemoTX\textsuperscript{15} Assay System. Within 20 h, cells migrated through the pores (black triangle) of the membrane towards a gradient of synovial fluid and firmly attached underneath the membrane (A). Cells were not evident in negative controls (B).
recruitment of mesenchymal progenitors. Also, the short-term observation period of 20 h may not resemble the in vivo situation, where signals might be present over weeks.

Apparently, the osteoarthritic environment does not impair cell migration. From this point of view, osteoarthritis may not be a contraindication for microfracture. In contrast, in an inflammatory environment mediated by, e.g., RA, the inflammatory response might lead to less cell migration and reduced or deferred tissue regeneration. This should be taken into account when treating cartilage defects in inflamed joints. The study shows that not only growth factors from the spongyous part of the joint might induce cell recruitment but also synovial fluid. In contrast to the cell recruiting capacity of synovial fluid obtained from normal or osteoarthritis donors, the chemotactic potential of rheumatoid arthritis synovial fluid to recruit mesenchymal progenitors is significantly reduced. The chemotactic activity of SF obtained from normal donors and from patients with degenerative joint diseases suggests that SF is actively involved in the migration of progenitors in cartilage defects after microfracture.
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REFERENCES

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