

Osteoarthritis and Cartilage



Multicolor flow cytometry-based cellular phenotyping identifies osteoprogenitors and inflammatory cells in the osteoarthritic subchondral bone marrow compartment



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SUMMARY

Purpose: The cellular component of subchondral bone is thought to be responsible for aberrant bone remodeling in osteoarthritis (OA). Direct phenotypical analysis of the cellular compartment is critical to better understand the OA disease process. This study provides proof-of-principle for flow cytometry-based phenotyping of isolated subchondral trabecular bone (STB) marrow cells without prior use of cell culture techniques.

Methods: Tibial plateaus were obtained from OA patients undergoing total knee arthroplasty. Subchondral bone chips were digested with collagenase 1A and single cell suspensions were directly phenotyped using flow cytometry. Cells were analyzed for the expression of alkaline phosphatase (ALP) as osteoblast/osteoprogenitor marker and monocyte/macrophage markers (CD14, CD68, HLA-DR, CD115).

Results: MTT staining revealed abundant viable cells in the bone marrow compartment of STB prior to digestion, which were efficiently released by collagenase. Within the CD45-negative cell fraction, approximately 20% of the cells were positive for the early osteoblast/osteoprogenitor marker ALP. Within the CD45+ hematopoietic cell fraction, the majority of cells were of monocytic origin (>80%) displaying strong surface expression of CD14. Discrete macrophage populations (CD14+/HLA-DR+/CD68+) and putative osteoclast progenitors (CD45+/HLA-DR-/CD115+) were unequivocally identified. Osteoblast, macrophage and osteoclast progenitor presence in the subchondral bone unit (SBU) was confirmed by (immuno)histochemical staining for osteocalcin, CD68 and tartrate-resistant acid phosphatase, respectively.

Conclusions: Flow cytometric analysis is a valuable methodology to study the cellular compartment of STB marrow. This method provides a proof of principle that the whole resident cell population can be directly phenotypically characterized without the prior use of cell culture techniques.

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Introduction

The subchondral bone unit (SBU) is comprised of the subchondral cortical plate and the directly subjacent subchondral

trabecular bone (STB) and marrow spaces. Clinically, it is well established that the molecular crosstalk between the cells in the SBU and the overlying cartilage plays a pivotal role in osteoarthritis (OA) disease progression and, potentially, induction^{1,2}. A hallmark of OA progression is osteosclerosis of the subchondral bone and it has recently been demonstrated that this shift towards increased bone formation results from an uncoupling of the activities of osteoblasts and osteoclasts within the trabecular marrow compartment of the SBU³. As the cellular component of the SBU is thought to be responsible for the structural changes in this tissue⁴, direct phenotypical analysis of the subchondral trabecular marrow compartment is critical to better understand the OA disease process.

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Current techniques to analyze cellular components of the SBU, including histology, explant outgrowth cultures and real time-PCR, have helped to identify and characterize the pathological bone remodeling in the osteoarthritic SBU, but several limitations exist, e.g., loss of cell types, mix of subpopulations, changes due to *in vitro* culture methods. Advanced flow cytometry techniques have been used to phenotypically characterize and isolate cells from a variety of soft tissues, including outgrowth cells from bone fragments, but to our knowledge it has never been demonstrated on cells directly isolated from the subchondral trabecular marrow compartment^{5–7}. Recent developments in novel fluorescent markers combined with technological advancements on flow cytometer capacities now allow users to simultaneously identify and characterize multiple complex cell phenotypes⁸. The ability to further separate cells based on phenotype and compare between healthy and diseased tissues allows for a concerted analysis of cellular functions and mechanisms.

In the present study, we assessed the feasibility of using flow cytometry as a tool to identify individual cell populations released by collagenase digestion from the marrow compartment of human subchondral bone from osteoarthritic knee tibial plateaus.

Materials and methods

Patients

Tibial plateaus were collected after written informed consent from five patients (three female, two male, mean age 68 ± 9 years) undergoing total knee arthroplasty. The study protocol was approved by the Ethics Committee of Basel (No. 147/12).

Marrow cell extraction

Freshly obtained tibial plateaus were rinsed twice in PBS and cartilage was carefully dissected from the subchondral cortical plate under a stereomicroscope (Leica, Germany). Sagittal bone slices (1 mm width, approximately 1 g wet weight in total) were digested in 8 mL α MEM supplemented with 0.6 mg/ml clostridial collagenase IA (Sigma, Switzerland). Samples were digested on an orbital shaker set to 60 rpm for 4 h at 37°C. Thereafter the digestion medium was collected and bone pieces were thoroughly rinsed once with PBS, which was then added to the cell suspension. Where indicated, CD45+ cells were isolated by magnetic bead separation following manufacturer's instructions (EasySep human CD45 depletion kit, Stemcell Technologies, USA).

MTT staining

Pre- and post-digest bone chips were stained for the presence of viable cells with 50 μ g/ml of MTT (Sigma) diluted in DMEM with no phenol red (Gibco, USA) for 1 h at 37°C. After rinsing with PBS, samples were imaged using a stereomicroscope (Leica, Germany).

Antibodies

Antibodies used for flow cytometric analysis were PE-conjugated anti-CD115 (9-4D2-1E4), PerCP/Cy5.5-conjugated anti-HLA-DR (L243), APC-conjugated anti-CD68 (Y1/82A), PE/Cy7-conjugated anti-CD14 (HCD14) and Brilliant Violet 421-conjugated anti-CD45 from Biolegend (UK). PerCP/Cy5.5 anti-alkaline phosphatase (B4-78) was from BD Pharmingen (USA). FITC-conjugated anti-CD45 (HI30) was from Tonbo Biosciences (USA). The mouse monoclonal antibodies used for immunohistochemical analysis were anti-human CD68 (PG-M1, Dako, Switzerland) and anti-human osteocalcin (OCG3, Abcam, UK).

Multicolor flow cytometric analysis

The cell suspension was centrifuged at 350 g for 5 min, the pellet resuspended in FACS buffer (PBS, 2 mM EDTA, 0.1% BSA) and incubated for 10 min in Human TruStain FcX (Biolegend, USA) to block nonspecific Fc receptor binding. Cell surface markers were stained for 30 min at room temperature in FACS buffer using antibody dilutions according to the manufacturer's instructions. Thereafter, cells were fixed and permeabilized in Flow Cytometry Fixation/Permeabilization Buffer I (R&D Systems) and stained intracellularly with anti-CD68. Samples were stained with DAPI (3 μ M in FACS buffer) for 15 min to distinguish cells from debris. Single-stained compensation beads (BD Biosciences) were used for multicolor compensation. Positive and negative selection gates were set using fluorescence minus one controls and unstained cells, respectively. Cells were analyzed on a LSRFortessa (BD Biosciences). Data were analyzed using FlowJo v10.0.7 (Tree Star Inc., USA).

Histology and immunohistochemistry

Formalin-fixed, decalcified tibial plateaus were processed for cryosectioning as described previously⁹. Sagittal cryosections (12 μ m) were stained with hematoxylin and eosin for analysis of marrow morphology. After antigen retrieval (10 mM citrate buffer pH 6.0 at 70°C for 30 min) cryosections (12 μ m) were stained with anti-CD68 (1:100) at room temperature for 1 h. Osteocalcin staining was performed overnight without antigen retrieval at a dilution of 1:200. Primary antibodies were visualized using a mouse-specific DAB/HRP (ABC) Detection IHC Kit (Abcam, UK). Tartrate-resistant acid phosphatase staining was performed by incubating cryosections for 1 h at 37°C in staining buffer containing 0.1 M acetate pH 5.2, 50 mM sodium tartrate, 0.1 mg/ml naphthol AS-MX and 0.3 mg/ml fast red LB violet salt (Sigma).

Results

Collagenase digestion releases CD45-negative and CD45-positive marrow cell fractions from the osteoarthritic STB

The marrow compartment of the STB in osteoarthritic tibial plateaus is histologically characterized by high cellularity and fibrous tissue (Fig. 1A). MTT staining of trimmed bone pieces revealed abundant viable cells in the marrow compartment prior to collagenase IA digestion. The vast majority of marrow-residing cells were released after four hours of digestion. (Fig. 1B). The obtained cells were largely viable (not shown) and their number sufficient for further testing. The analysis of flow cytometry data consisted in a first step of discriminating DAPI positive events, identified as nucleated cells, from DAPI negative bone debris. Doublets and artifacts were removed by gating only events in the diagonal cluster of a forward scatter height (FSC-H) vs forward scatter area (FSC-A) plot. The presence of cell types of both hematopoietic and non-hematopoietic origin was revealed from staining against CD45 (Fig. 1C).

Monocytes, macrophages and osteoclast progenitors reside in the CD45+ marrow cell fraction

The precise identity of different cell populations within the hematopoietic cell fraction was determined by additional markers. The majority (>80%) of CD45+ cells were identified as CD14+/CD68– monocytes and part were double positive CD14+/CD68+ macrophages. Complementary immunostaining for CD68 in histological tissue sections confirmed the presence of mononuclear macrophages in the osteoarthritic subchondral trabecular marrow

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