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Mesenchymal stromal/stem cells markers in the human bone marrow

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Abstract

Background aims. Mesenchymal stromal/stem cells (MSCs) can be isolated from human bone marrow (BM), expanded *ex vivo* and identified via numerous surface antigens. Despite the importance of these cells in regenerative therapy programs, it is unclear whether the cell membrane signature defining MSC preparations *ex vivo* is determined during culture or may reflect an *in vivo* counterpart. BM-MSC phenotype *in vivo* requires further investigation. *Methods.* To characterize cells in their natural BM environment, we performed multi-parametric immunohistochemistry on trabecular bone biopsy specimens from multiple donors and described cells by different morphology and micro-anatomic localization in relationship to a precise pattern of MSC antigen expression. *Results.* Microscopically examined high-power field marrow sections revealed an overlapping *in vivo* expression of antigens characterizing *ex vivo* expanded BM-MSCs, including CD10, CD73, CD140b, CD146, GD2 and CD271. Expanding this panel to proteins associated with pluripotency, such as Oct4, Nanog and SSEA-4, we were able to identify different cellular populations in the human trabecular bone and BM expressing different progenitor cell markers. *Conclusions.* Targeting several multipotency and pluripotency markers, we found that the BM contains identifiable and distinct progenitor cells further justifying their introduction for a wide range of applications in regenerative medicine.

Key Words: *bone marrow, histology, in vivo, markers, MSCs, pluripotency, stromal cells*

Introduction

Bone marrow (BM) mesenchymal stromal/stem cells (MSCs) are rare elements (<0.01%) with promising therapeutic properties based on both multi-lineage differentiation capacities and secretion of paracrine factors (1,2). MSCs are currently applied in regenerative medicine (3,4), and emerging studies demonstrate their potential in immunotherapy (5–7). Because of variations of isolation techniques and variable *in vitro* culture conditions, comparable analyses of MSC sub-populations *in vitro* have been difficult to implement (8,9). Minimal criteria, such as adherence to plastic surfaces, expression of CD105, CD73 and CD90, lack of expression of hematopoietic markers and *in vitro* differentiation into mesenchymal tissues, were established to define MSCs (10). Although these criteria have been established to help standardization within studies on MSCs, it is recognized that these are heterogeneous cell preparations sharing a spindle-fibroblastoid phenotype *in vitro* (11,12). More recently, novel molecules have been described to identify MSC sub-populations, such as the ganglioside GD2, which seems to be exclusively expressed on MSCs in healthy marrow (1,13–16). Extensive investigations on human BM-MSC sub-populations *in vitro* have been performed for decades, but there is still little knowledge on features of MSCs in their natural *in vivo* environment, such as the BM. However, this knowledge is a prerequisite for sophisticated approaches of BM-MSC isolation and culture *in vitro* as well as for the development of cellular therapeutics based on prospectively isolated BM-MSCs. Elucidating this relevant knowledge in MSC research would broaden our understanding of mesenchymal cell entities harbored in the human BM and isolated therefrom and would fortify the basis for future clinical applications of MSCs. Performing multiparametric

immunohistochemistry (IHC) on multiple specimens of trabecular bone from human femora, we addressed antigen expression patterns considering 12 antibodies that were selected according to the criteria summarized in Supplementary Table I. In this paper, we provide qualitative analyses with a rich iconography of elements expressing MSC markers *in vivo* with different morphology and micro-anatomic distribution, additionally revealing the expression of pluripotency markers within human marrow cells.

Methods

BM-MSC isolation and culture

BM was collected under sterile conditions from patients (n = 25) without metabolic or neoplastic diseases during orthopedic operations as described previously (12). All patients gave written informed consent for BM isolation. The study was approved by the local institutional review board. Briefly, 5−10 mL of BM was collected into a sterile heparinized syringe from each subject. Whole BM was diluted in an equal volume of phosphate-buffered saline (Lonza, Walkersville, MD, USA) and laid over 20 mL of a density gradient (Lymphoflot; Biotest, Dreieich, Germany) for subsequent centrifugation (20 min, 1000*g*, without brake at 20°C). Mononuclear cells were harvested, washed with standard culture medium composed of α-MEM medium (Lonza), 100 U/mL penicillin-streptomycin (Lonza) and 10% pooled human serum (blood group AB), transferred to 75-cm^2 or 185-cm^2 culture flasks (Nunc GmbH Co Kg, Wiesbaden, Germany) at 10^5 cells per cm^2 and incubated at 37°C in humidified atmosphere at 5% carbon dioxide using standard culture medium. After 24 h, the non-adherent cells were removed, and the adherent cells were further cultured in standard culture medium. Reaching sub-confluency, the cells were detached from flasks with trypsin/ethylenediamine tetraacetic acid (Lonza), washed with phosphate-buffered saline and transferred to new flasks at a density of 10^3 cells/cm^2.

Characterization of surface antigens by flow cytometry

For flow cytometry, MSCs were detached from flasks and incubated for 30 min in the dark at 4°C with primary antibodies followed by secondary antibodies where applicable. Cells were suspended in 50 μL of phosphate-buffered saline with 1% bovine serum albumin (Sigma-Aldrich, St Louis, MO, USA), 0.1% fetal calf serum (FCS, Lonza) and 0.1% sodium azide (Sigma-Aldrich). The antibodies were used at 0.1 μg/10^5 cells or according to the manufacturer's instructions. The cell suspensions in 300 μL were analyzed for 10^4 events (fluorescence-activated cell sorter scan with BD CellQuest Pro software; BD Biosciences, San Jose, CA, USA). 7-Amino-actinomycin D (BD Biosciences) was used to exclude dead cells. The introduced monoclonal antibodies were mouse anti-human phycoerythrin-conjugated or non-labeled anti-CD10, anti-CD31, anti-CD34, anti-CD45, anti-CD73, anti-CD140b, anti-CD146, anti-GD2 (BD Biosciences) and anti-CD271 (Miltenyi Biotec GmbH, Bergisch-Gladbach, Germany); phyco-erythrin-conjugated or non-labeled IgG1 and IgG2a antibodies (BD Biosciences) were used as isotype matched controls. Secondary antibody was a polyclonal phycoerythrin-conjugated goat anti-mouse immuno-globulin (BD Biosciences). Staining and flow cytometry analyses were performed at the end of passage 1.

Collection of bone biopsy specimens

Bone samples were obtained from femoral heads of 10 patients (five women and five men) at a median age of 55 years (±7.6 years standard deviation) during hip arthroplasty surgery. All patients were without metabolic or neoplastic diseases. All patients gave written informed consent, and the study was approved by the local institutional review board. After collection, the samples were immediately cut into cubes of 3 cm^3 in size and fixed in 10% neutral buffered formalin solution (Sigma-Aldrich) for at least 24 h.

IHC staining

Fragments of human femoral hip were fixed, decalcified and paraffin-embedded. After heat-induced (citrate buffer) or enzymatic antigen retrievals (Table I), 4-μm bone sections underwent either single or double immunohistochemical stains using rabbit and mouse primary antibodies. The main retrieval conditions and the list of all antibodies used and their dilutions are specified in Table I. Goat anti-rabbit or anti-mouse biotinylated secondary antibodies (1:200; Vector Laboratories, Burlingame, CA, USA) were used, and after incubation with VECTASTAIN ABC (Vector Laboratories), horseradish peroxidase-labeled antibodies were visualized by NovaRed (in red; Vector Laboratories), DAB (in brown; Vector Laboratories) or DAB-nickel (black; Vector Laboratories) where applicable. All slides were counterstained with Harris Hematoxylin (Bio-Optica Milano S.p.a, Milan, Italy). Stained slides were examined on a Zeiss Axioskop (Germany) with 40×/0.75 NA dry objective at room temperature. Photomicrographs were acquired with AxioCam ICc3 color camera (MicroImaging GmbH, Carl Zeiss Group, Jena, Germany) and AxioVision software (Carl Zeiss Imaging Solution GmbH, Hallbergmoos, Germany). Images were analyzed and processed by AxioVision LE (Carl Zeiss Imaging Solution