Experimental Hematology

Experimental Hematology 2013;41:558–566

Molecular and functional characterization of human bone marrow adipocytes

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(Received 12 December 2012; revised 30 January 2013; accepted 3 February 2013)

Adipocytes are a cell population largely located in the human bone marrow cavity. In this specific microenvironment where adipocytes can interact with a variety of different cells, the role of fat is mainly unknown. To our knowledge, this report is the first to characterize mature adipocytes isolated from human bone marrow (BM-A) molecularly and functionally to better understand their roles into the hematopoietic microenvironment. Healthy BM-A were isolated after collagenase digestion and filtration. We studied the morphology of BM-A, their gene expression and immunophenotypic profile and their functional ability in the hematopoietic microenvironment, comparing them with adipocytes derived from adipose tissue (AT-A). BM-A showed a unilocular lipid morphology similar to AT-A and did not lose their morphology in culture; they showed a comparable pattern of stem cell-surface antigens to AT-A. In line with these observations, molecular data showed that BM-A expressed some embryonic stem cells genes, such as Oct4, KLf4, c-myc, Gata4, Tbx1, and Sox17, whereas they did not express the stem cell markers Sox2 and Nanog. Moreover, BM-A had long telomeres that were similar to bone marrow mesenchymal stem cells. Notably, BM-A supported the survival and differentiation of hematopoietic stem cells in long-term cultures. These results showed that BM-A are stromal cells with a gene expression pattern that distinguished them from AT-A. BM-A showed stem cell properties through their hematopoietic supporting function, which was certainly linked to their role in the maintenance of the bone marrow microenvironment. Depending on specific demands, BM-A may acquire different functions based on their local environment.

The bone marrow (BM) microenvironment contains many different types of cells. Hematopoietic and stromal stem cells are more highly studied than BM fat, which is an abundant component in adult BM [1]. Many studies have reported that active hematopoietic BM, called red *bone marrow*, declines with age and is gradually converted to fatty marrow, called yellow *bone marrow*, from the periphery toward the axial skeleton [2]. With age, nonhematopoietic fatty marrow progressively predominates and fills the entire marrow cavity through a dynamic and reversible process [3].

Adipocytes represent a terminally differentiated cell population with a specific morphology characterized by the presence of a single, large cytoplasmic lipid droplet that accounts for approximately 90% of the cellular volume [4]. The role of fat in the BM microenvironment is largely unknown, and these cells have not been well characterized for their molecular and functional properties, contrary to adipose tissue adipocytes (AT-A) [5,6]. Recently, we demonstrated that mature human adipocytes isolated from the omental and subcutaneous adipose tissue depots have stem cell–like properties that could explain their plasticity and functional features [5–8].

We studied bone marrow adipocytes (BM-A) to try to understand their functions in the hematopoietic microenvironment and to compare their stemness with AT-A. Accordingly, we isolated and cultured BM-A in vitro and characterized these cells for their morphologic, molecular, and immunophenotypic properties. This study is the first

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Supplementary data related to this article can be found online at http://dx.doi.org/10.1016/j.exphem.2013.02.005.

http://dx.doi.org/10.1016/j.exphem.2013.02.005

to provide a detailed characterization of BM-A, studying the gene expression profile of genes involved in white and brown adipogenic lineage, stemness and lineage reprogramming, telomere length, and hematopoietic supporting capabilities. In addition to hematologic effects, information on marrow fat is of potential relevance to understand the fat plasticity. Indeed, this phenomenon is not limited to the adipose tissue, but could be demonstrated also in many fat-rich organs, such as parotid glands, parathyroid glands, thymus, lymph nodes, and the pancreas.

Methods

Isolation and culture of mature adipocytes

Bone marrow was harvested from the iliac crest of healthy BM donors (n = 9; median age, 45 years; range, 29–55 years) after obtaining written informed consent and in accordance with the guidelines of the local ethical committee (300-DG). Bone marrow (5–10 mL) from each donor was diluted with PBS and layered onto Ficoll (MP Biomedicals, Iukirch, France). The Ficoll gradient was spun at 370 g for 30 min at 4°C. The floating adipocytes and the interphase mononuclear cells were collected. Subcutaneous fat tissues (5–10 g) were harvested from patients undergoing abdominal plastic surgery (n = 10; median age, 49 years; range, 35–60 years) after obtaining written informed consent and in accordance with the guidelines of the local ethical committee (300-DG). After Ficoll separation, the BM samples were treated with a solution of 1 mg/mL of type I collagenase (Gibco; Invitrogen, Milan, Italy) and 2% human albumin at 37°C for 1 hour, whereas adipose tissue samples were minced into smaller pieces and incubated with 3 mg/mL of type I collagenase and 2% human albumin at 37°C for 2 hours. After digestion, the samples were filtered through a 200-μm nylon sieve to obtain mature adipocytes that were free of other cells. The filtered cells were washed four times with Dulbecco's modified Eagle's medium (DMEM; Biological Industries, Milan, Italy) and centrifuged at 250 rcf for 5 min. Only the floating cell fraction was collected after each centrifugation step. Cells were seeded for ceiling culture as previously described [7,9] with DMEM supplemented with 20% fetal bovine serum (Stem Cell Technologies, Vancouver, BC, Canada) and cultured at 37°C and 5% CO_2 or collected for molecular assays.

Immunofluorescence of isolated adipocytes

Confocal microscopy optical sectioning and computer-assisted image reconstruction of isolated adipocytes allowed us to exclude the presence of small, undifferentiated cells attached to the surface of the analyzed adipocytes. Isolated adipocytes were fixed in 4% paraformaldehyde, incubated with rabbit anti-perilipin (provided by Dr. A.S. Greenberg, Tufts University, Medford, MA, USA; 1:50 in PBS) and stained with FITC-linked secondary antibodies (Jackson ImmunoResearch, West Grove, PA, USA; 1:100 in PBS). Images were taken in the green channel. TOTO-3 iodide (Molecular Probes, Invitrogen, Monza, Italy; 1:5000 in PBS) was used as the nuclear counterstain and visualized in the blue channel. We analyzed at least 100 adipocytes from each suspension. Images were sequentially obtained from two channels using a pinhole of 1:1200.

Electron microscopy

Bone marrow biopsy specimens collected for diagnostic reasons at electron microscopy service of our hospital were selected on the base of their adipocyte content. Solid small fragments of BM were immediately fixed in 2% glutaraldehyde−2% formaldehyde in 0.1 mol/L phosphate buffer (pH 7.4) for at least 4 hours, postfixed in a solution of 1% osmium-tetroxide, dehydrated in acetone, and finally embedded in epoxy resin. Significant areas from semithin resin sections (1.5 μm) were selected to be studied at electron microscopy level; thin sections (∼60 nm) obtained with an MT-X Ultratome (RCM, Tucson, AZ, USA) were mounted on copper grids, stained with lead citrate, and examined with a CM10 transmission electron microscope (Philips, Eindhoven, The Netherlands).

Immunophenotype analysis

All BM-A and AT-A samples were characterized by flow cytometry after isolation. Cells were stained with fluorescein isothiocyanate (FITC)-, phycoerythrin (PE)-, or peridin chlorophyll protein (PerCP)-conjugated antibodies against CD90 (Thy-1; BD Pharmingen, San Diego, CA, USA), CD105 (endoglin; BD Pharmingen), CD271 (NGFR; Miltenyi Biotech, Cologne, Germany), CD117 (c-kit; Miltenyi Biotech), CD34 (hematopoietic progenitor cell antigen; BD Biosciences, Franklin Lakes, NJ), CD133 (hematopoietic stem cell antigen; Miltenyi Biotech), CD45 (leukocyte common antigen; BD Biosciences), and CD31 (platelet endothelial cell adhesion molecule; BD Biosciences). FITC-, PE- (Dako, Glostrup, Denmark), and PerCP- (BD Pharmingen) negative isotypes were used as control antibodies. Cells were incubated with primary antibodies at 4°C for 30 min. Thereafter, cell fluorescence was evaluated using a FACSCalibur flow cytometer (Becton Dickinson). The data were analyzed using CellQuest software.

Adipogenic lineage gene expression profile

Total RNA was extracted using the RNeasy Plus Micro Kit (Qiagen, Milan, Italy) according to the manufacturer's instructions. The purity of the RNA was confirmed by determining the 260 nm/280 nm absorbance ratio (>1.8). For each sample, 1 μg of total RNA was reverse transcribed as described previously [7]. To quantify adipocyte lineage gene expression of adiponectin (NM_004797.2; 173 bp), fatty acid binding protein four (aP2, NM_001442.2; 133 bp), peroxisome proliferators-activated receptor gamma coactivator 1 alpha (PGC1 alpha, NM_013261.3; 164 bp), leptin (LEP, NM_000230.2; 150 bp), cell death–inducing DFFA-like effector a (CIDEA, NM_001279.3; 167 bp), deiodinase type II (DIO2, NM-013989.4; 196 bp), real-time polymerase chain reaction (PCR) assays were performed with BM-A and AT-A samples. The transcript levels of these genes were normalized to the expression of the constitutive gene IPO8 (NM_006390.3; 185 bp) and an endogenous calibrator (BM–derived mesenchymal stem cells [BM-MSCs]) following the $2^{-\Delta\Delta Ct}$ method [10]. The SYBR Select Master Mix (Life Technologies, Monza, Italy) and specific primers were optimized for the ABI 7500 Fast system (Applied Biosystems, Foster City, CA, USA) in a 20 μL mixture, containing 10 μL of MasterMix, 10 μmol/L of each primer and 2 μL of cDNA template. After 20 sec at 50°C and 10 min at 95°C, 40 cycles of denaturation, 15 sec at 95°C, and annealing/extension for 1 min at 60°C were run. The MeltCurve Stage was performed using one cycle at 95°C for 15 sec, 60°C for 1 min, 95°C for 30 sec, and 60°C for 15 sec. Reverse transcriptase PCR assays were performed to quantify the expression