Biochemical and Biophysical Research Communications 378 (2009) 539–544

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

Flow cytometric and immunohistochemical detection of *in vivo* BrdU-labeled cells in mouse fat depots

Jaroslaw Staszkiewicz [a,b], Jeffrey Gimble [a], Courtney Cain [a], Marilyn Dietrich [c], David Burk [a], Heather Kirk-Ballard [a], Barbara Gawronska-Kozak [a,*]

[a] Pennington Biomedical Research Center, Louisiana State University System, 6400 Perkins Rd., Baton Rouge, LA 70808, USA
[b] Department of Animal Physiology, Warmia & Mazury University, Olsztyn, Poland
[c] LSU Flow Cytometry Facility, School of Veterinary Medicine, Louisiana State University, Baton Rouge, LA, USA

ARTICLE INFO

Article history:
Received 30 October 2008
Available online 3 December 2008

Keywords:
Label-retaining cells
BrdU
Flow cytometry
Sca-1
WAT
BAT
Adipocytes

ABSTRACT

This study has determined the natural frequency and localization of progenitor/stem cells within fat depots *in situ* based on their ability to retain DNA nucleotide label (BrdU). Neonate and mature male C57BL6/J mice were injected intraperitoneally with BrdU- and label-retaining cells (LRC) were quantified in fat depots by immunohistochemical, immunofluorescent, and flow cytometric methods. In neonates, LRC constituted 27% of the cells in inguinal fat (iWAT) and 65% in interscapular brown fat (BAT) after Day 10 and 26% of the cells in epididymal fat (eWAT) after Day 28. After 52 days, the LRC accounted for 0.72% of iWAT, 0.53% of eWAT and 1.05% of BAT, respectively. The BrdU-labeled cells localized to two areas: single cells distributed among adipocytes or those adjacent to the blood vessels wall. In mature C57BL6/J mice, flow cytometric analysis determined that a majority of the LRC were also positive for stem cell antigen-1 (Sca-1).

Weight gain associated with obesity is characterized by an increase in adipose mass that is achieved by two processes: hypertrophy of existing fat cells and hyperplasia throughout generation of new adipocytes from the pool of tissue resident stem cells. Hypertrophy of adipocytes through a positive energy balance has been well documented in humans and animal models [1]. While some studies have suggested that WAT expansion during adulthood also results from an increased number of adipocytes [2,3], other studies point out that the number of fat cells stays constant in both lean and obese adults as recently shown for human adipose tissues [4]. A platform where this controversy meets is *in vitro* vs. *in vivo* studies. The tremendous progress in *in vitro* techniques allows for the isolation and culture of stem cells/progenitor cells from adult tissues and the ability of adult stem cells to differentiate into several lineages, to display clonogenic potentials, and to express transcriptional factors related to their undifferentiated/pluripotent stage has been well documented [5–8]. Although many features of the molecular properties of these adult stem cells have been described, a well-defined signature for their presence is still missing [1].

The lack of precise molecular markers for adult stem cells is a limiting factor of *in vivo* studies and *in vivo* methodologies that aim to overcome this obstacle are based on the slow-cell cycling feature of stem cells [9–14]. The principle of the method posits that *in vivo* labeling through incorporation of [^{3}H]thymidine or 5-bromo-2′-deoxyuridine (BrdU) into cells' DNA during the early postnatal development allows for the detection of slow-cycling/stem cells through the retention of the label in adult animals. This approach has been successfully used to identify label-retaining cells (LRC)/stem cells in epithelial compartment as in skin [12], bladder [10], colon [14], and endometrium [11], and in some of the mesenchymal compartments: myometrium [13] and kidney [9].

Adipose tissues contain a population of adult/somatic stem cells that have been isolated from human and animals [5,15]. These cells, termed adipose-derived stem or stromal cells (ASC), are self renewing and can differentiate along several mesenchymal tissues lineages including adipocytes, osteoblasts, myocytes, chondrocytes, endothelial cells, and neurocytes. Whereas ASC are very well characterized *in vitro*, there is little knowledge of their natural distribution and localization within fat tissues *in vivo*. Elegant studies performed by Hirsh and co-workers in the 1970s and early 1980 [2,16] detected a small population of cells residing in the stromal-vascular fraction of adipose tissues that can take up [^{3}H]thymidine. The authors concluded that these cells "may be an adipocyte progenitor or may have some other unknown function"

* Corresponding author. Fax: +1 225 763 0273.
E-mail address: KozakB@pbrc.edu (B. Gawronska-Kozak).
URL: http://www.pbrc.edu (B. Gawronska-Kozak).

0006-291X/$ - see front matter © 2008 Elsevier Inc. All rights reserved.
doi:10.1016/j.bbrc.2008.11.092

[16]. Following in the footsteps of Hirsh's studies, the current article describes the *in vivo* localization and characteristics of LRC in adipose tissues. We demonstrate the existence of LRC/stem cells in fat depots that are localized to two compartments: those close to the blood vessels wall and those sparsely distributed among adipocytes. Moreover, we show that BrdU-retaining cells are stem cell antigen-1 (Sca-1) positive and hematopoietic lineage (CD45/CD4) negative. These findings confirm and extend our previous studies showing that Sca-1 is a biomarker for ear mesenchymal stem cells (EMSC) with adipogenic potential [17].

Materials and methods

Experimental animals and BrdU labeling. All experiments involving animals were approved by the Pennington Biomedical Research Center Institutional Animal Care and Use Committee in accordance with NIH guidelines. We used the BrdU labeling approach to label and then identify/quantify LRC in fat depots. In the first experiment neonate, 3.5-day-old male C57BL/6J mice ($n = 10$) were injected intraperitoneally with BrdU at a concentration of 50 μg/g of body weight (Sigma Co., St. Louis, MO) twice daily, at 7.00 am and 6.00 pm for 3 consecutive days (total 7 injections). Control animals (littermates; $n = 5$) were injected with saline. Chase periods for BrdU-labeled fat tissues were 10, 28 or 39, and 52 days. Tissues were harvested and analyzed for BrdU containing nuclei by immunohistochemistry and immunofluorescence.

The second experiment was performed on adult animals and involved the flow cytometric detection of BrdU-retaining cells in the stromal-vascular fraction of fat depots and in bone marrow (BM) cells. Male 4-month-old C57BL/6J mice were intraperitoneally injected with BrdU ($n = 6$) or saline (control $n = 4$). The BrdU dose and schedule of injections were the same as in the first experiment. Animals were euthanized 24 h ($n = 6$) after the last injection of BrdU. Epididymal and inguinal fat depots were collected, minced, and digested with collagenase class I (2 mg/ml; Worthington Biochemical Corp., Freehold, NJ). Bone marrow cells were collected from tibia and femurs. Cells were suspended in red blood cell lysing buffer (Sigma Co., St. Louis, MO) to remove erythrocyte contamination before analyzing by flow cytometry.

Histological detection of BrdU-labeled cells. Euthanized animals were perfused with PBS via cardiac puncture followed by 4% paraformaldehyde. Dissected fat depots were embedded in paraffin and sectioned at 5 μm.

Immunohistochemistry. To denaturate DNA dewaxed sections were treated with 1 N HCl at room temperature for 10 min, then with 2 N HCl at 37 °C for 20 min followed by incubation in 0.1 M borate buffer. Primary anti-BrdU antibody (rat anti-mouse ab6326 Abcam) at a concentration of 1:400 was applied overnight at 4 °C.

For immunohistological antibody binding detection ABC complex was applied (Vectastain ABC kit, Vector Laboratories, Inc., Burlingame, CA). Two types of controls were performed: (a) the primary antibody was substituted with non-specific immunoglobulin G (IgG) during the procedure, and (b) fat depot sections from saline treated animals were subjected to BrdU detection. Samples were counterstained with hematoxylin. Sections were visualized with a Zeiss microscope (Axioskop 40) using Plan-Neofluor 10× objective and photographed with a Kodak digital camera (DC290 Zoom). Quantitative analyses of immunopositive cells were made using Metamorph software. For LRC quantitation, adipocytes and BrdU-positive cells were counted from 5-fields and the label-retaining cells expressed as a percentage of total adipocytes counted. BrdU-retaining cells in brown fat tissues were expressed as a percentage of total nuclei counted.

For immunofluorescence detection of BrdU-labeled cells goat anti-rat antibodies conjugated to Alexa Fluor 488 (Molecular Probes, Eugene, OR) followed overnight incubation with primary anti-BrdU antibody were applied. BrdU expression was imaged using an Everest imaging system (Intelligent Imaging Innovation, Inc., Denver, CO) based on a Zeiss Meta 510 confocal microscope.

BrdU flow cytometry assay. Cells from the stromal-vascular fraction of fat depots or BM were first incubated with phycoerythrin (PE) conjugated anti-Sca-1 antibodies (BD Pharmingen San Diego, CA). Similar cells suspensions were incubated with PE conjugated anti-CD45 and anti-CD4 antibodies (BD Pharmingen San Diego, CA). Next cells were fixed, treated with DNase to expose BrdU epitopes, and incubated with FITC-conjugated anti-BrdU antibodies following instruction of the manual (FITC BrdU Flow Kit BD Pharmingen San Diego, CA). The flow cytometry assay was performed using a FACScan flow cytometer (Becton Dickinson, San Jose, CA) and data were analyzed with a Macintosh G5 workstation (Apple Computer, Cupertino, CA), which contains Cellquest graphics software (Becton Dickinson, San Jose, CA) for data acquisition and analysis. Gates were always set using cells isolated from animals that received injections of saline instead of BrdU, but the samples were processed and stained alongside the experimental samples. The data were expressed as a percent of BrdU, Sca-1, and CD4/CD45 positive cells per gated cells. FACS plots and data are representative of three separate experiments.

Results

BrdU-retaining cells during postnatal fat depots development

In our first experiment, we administered BrdU to 3.5-day-old mice for a period of 3 days. The fat depots were collected at Day 10, 28 or 39, and 52. In the mouse, adipose tissue is formed postnatally; subcutaneous iWAT first appears at about 1 day of age, while the visceral white fat depots appear at about 9 days of age [1,18]. On the other hand, BAT development starts during late gestation with developmental reaching a peak at Day 10 after birth [1,18]. Therefore, we collected BAT and inguinal white adipose tissues (iWAT) at Day 10 of postnatal life. At Day 28, 39, and 52 we collected BAT, iWAT, and epididymal white adipose tissues (eWAT).

Interscapular brown adipose tissue

At Day 10 of development quantitative analysis of BAT histological sections revealed that 65.76% ± 25.9% of total nuclei are positive for BrdU (Fig. 1A and I). To confirm that BrdU incorporation is localized to the nucleus we performed double-immunofluorescence staining using FITC-conjugated anti-BrdU antibodies together with DAPI staining (Fig. 1L). As expected, BrdU staining co-localized with DAPI stained nuclei (Fig. 1L). Immunofluorescent and immunohistochemical analyses showed that LRC were uniformly distributed through BAT (Fig. 1A and L). On Day 28 the number of BrdU-positive cells had decreased to 22.8% ± 2.3% (Fig. 1B and I), although the LRC distribution and intensity of staining were similar to that on Day 10. A significant decrease in number of cells positive for BrdU was observed at Day 52 (Fig. 1C and I) when 1.05% ± 0.6% of cells still retained intense staining for incorporated BrdU.

Inguinal white adipose tissue

To quantify the number of BrdU-retaining cells in white fat depots, we expressed them as a percentage of the number of adipo-